Hedgehog Antagonists, Methods and Uses Related Thereto

This application is based on U.S. provisional application 60/240,564, filed October 13, 2000, hereby incorporated by reference.

Background of the Invention

Pattern formation is the activity by which embryonic cells form ordered spatial arrangements of differentiated tissues. The physical complexity of higher organisms arises during embryogenesis through the interplay of cell-intrinsic lineage and cell-extrinsic signaling. Inductive interactions are essential to embryonic patterning in vertebrate development from the earliest establishment of the body plan, to the patterning of the organ systems, to the generation of diverse cell types during tissue differentiation (Davidson, E., (1990) Development 108: 365-389; Gurdon, J. B., (1992) Cell 68: 185-199; Jessell, T. M. et al., (1992) Cell 68: 257-270). The effects of developmental cell interactions are varied. Typically, responding cells are diverted from one route of cell differentiation to another by inducing cells that differ from both the uninduced and induced states of the responding cells (inductions). Sometimes cells induce their neighbors to differentiate like themselves (homeogenetic induction); in other cases a cell inhibits its neighbors from differentiating like itself. Cell interactions in early development may be sequential, such that an initial induction between two cell types leads to a progressive amplification of diversity. Moreover, inductive interactions occur not only in embryos, but in adult cells as well, and can act to establish and maintain morphogenetic patterns as well as induce differentiation (J.B. Gurdon (1992) Cell 68:185-199).

Members of the *Hedgehog* family of signaling molecules mediate many important short- and long-range patterning processes during invertebrate and vertebrate development. In the fly, a single *hedgehog* gene regulates segmental and imaginal disc patterning. In contrast, in vertebrates, a *hedgehog* gene family is involved in the control of left-right asymmetry, polarity in the CNS, somites and limb, organogenesis, chondrogenesis and spermatogenesis.

The first *hedgehog* gene was identified by a genetic screen in the fruit fly *Drosophila melanogaster* (Nüsslein-Volhard, C. and Wieschaus, E. (1980) Nature 287, 795-801). This

screen identified a number of mutations affecting embryonic and larval development. In 1992 and 1993, the molecular nature of the *Drosophila hedgehog* (*hh*) gene was reported (*C.F.*, Lee et al. (1992) Cell 71, 33-50), and since then, several *hedgehog* homologues have been isolated from various vertebrate species. While only one *hedgehog* gene has been found in *Drosophila* and other invertebrates, multiple *Hedgehog* genes are present in vertebrates.

The vertebrate family of *hedgehog* genes includes at least four members, e.g., paralogs of the single drosophila *hedgehog* gene. Exemplary *hedgehog* genes and proteins are described in PCT publications WO 95/18856 and WO 96/17924. Three of these members, herein referred to as Desert *hedgehog* (*Dhh*), Sonic *hedgehog* (*Shh*) and India hedge*hog* (*Ihh*), apparently exist in all vertebrates, including fish, birds, and mammals. A fourth member, herein referred to as tiggie-winkle *hedgehog* (*Thh*), appears specific to fish. Desert *hedgehog* (*Dhh*) is expressed principally in the testes, both in mouse embryonic development and in the adult rodent and human; India hedge*hog* (*Ihh*) is involved in bone development during embryogenesis and in bone formation in the adult; and *Shh*, which, as described above, is primarily involved in morphogenic and neuroinductive activities. Given the critical inductive roles of *hedgehog* polypeptides in the development and maintenance of vertebrate organs, the identification of hedgehog interacting proteins is of paramount significance in both clinical and research contexts.

The various *Hedgehog* proteins consist of a signal peptide, a highly conserved N-terminal region, and a more divergent C-terminal domain. In addition to signal sequence cleavage in the secretory pathway (Lee, J.J. et al. (1992) Cell 71:33-50; Tabata, T. *et al.* (1992) Genes Dev. 2635-2645; Chang, D.E. *et al.* (1994) Development 120:3339-3353), *Hedgehog* precursor proteins undergo an internal autoproteolytic cleavage which depends on conserved sequences in the C-terminal portion (Lee *et al.* (1994) Science 266:1528-1537; Porter *et al.* (1995) Nature 374:363-366). This autocleavage leads to a 19 kD N-terminal peptide and a C-terminal peptide of 26-28 kD (Lee *et al.* (1992) supra; Tabata *et al.* (1992) supra; Chang *et al.* (1994) supra; Lee *et al.* (1994) supra; Bumcrot, D.A., *et al.* (1995) Mol. Cell. Biol. 15:2294-2303; Porter *et al.* (1995) supra; Ekker, S.C. *et al.* (1995) Curr. Biol. 5:944-955; Lai, C.J. *et al.* (1995) Development 121:2349-2360). The N-terminal peptide stays tightly associated with the surface of cells in which it was synthesized, while the C-terminal peptide is freely diffusible both *in vitro* and *in vivo* (Porter et al. (1995) Nature 374:363; Lee *et al.* (1994) supra; Bumcrot *et al.* (1995) supra; Marti, E. *et al.* (1995) Development 121:2537-2547; Roelink, H. *et al.* (1995) Cell

81:445-455). Interestingly, cell surface retention of the N-terminal peptide is dependent on autocleavage, as a truncated form of HH encoded by an RNA which terminates precisely at the normal position of internal cleavage is diffusible *in vitro* (Porter *et al.* (1995) supra) and *in vivo* (Porter, J.A. *et al.* (1996) Cell 86, 21-34). Biochemical studies have shown that the autoproteolytic cleavage of the HH precursor protein proceeds through an internal thioester intermediate that subsequently is cleaved in a nucleophilic substitution. It is likely that the nucleophile is a small lipophilic molecule that becomes covalently bound to the C-terminal end of the N-peptide (Porter *et al.* (1996) supra), tethering it to the cell surface. The biological implications are profound. As a result of the tethering, a high local concentration of N-terminal *Hedgehog* peptide is generated on the surface of the *Hedgehog* producing cells. It is this N-terminal peptide which is both necessary and sufficient for short- and long-range *Hedgehog* signaling activities in *Drosophila* and vertebrates (Porter *et al.* (1995) supra; Ekker *et al.* (1995) supra; Lai *et al.* (1995) supra; Roelink, H. *et al.* (1995) Cell 81:445-455; Porter *et al.* (1996) supra; Fietz, M.J. *et al.* (1995) Curr. Biol. 5:643-651; Fan, C.-M. *et al.* (1995) Cell 81:457-465; Marti, E., *et al.* (1995) Nature 375:322-325; Lopez-Martinez *et al.* (1995) Curr. Biol 5:791-795; Ekker, S.C. *et al.* (1995) Development 121:2337-2347; Forbes, A.J. *et al.* (1996) Development 122:1125-1135).

HH has been implicated in short- and long-range patterning processes at various sites during *Drosophila* development. In the establishment of segment polarity in early embryos, it has short-range effects that appear to be directly mediated, while in the patterning of the imaginal discs, it induces long-range effects via the induction of secondary signals.

In vertebrates, several *hedgehog* genes have been cloned in the past few years. Of these genes, *Shh* has received most of the experimental attention, as it is expressed in different organizing centers, which are the sources of signals that pattern neighboring tissues. Recent evidence indicates that *Shh* is involved in these interactions.

The expression of *Shh* starts shortly after the onset of gastrulation in the presumptive midline mesoderm, the node in the mouse (Chang *et al.* (1994) supra; Echelard, Y. *et al.* (1993) Cell 75:1417-1430), the rat (Roelink, H. *et al.* (1994) Cell 76:761-775) and the chick (Riddle, R.D. *et al.* (1993) Cell 75:1401-1416), and the shield in the zebrafish (Ekker *et al.* (1995) supra; Krauss, S. *et al.* (1993) Cell 75:1431-1444). In chick embryos, the *Shh* expression pattern in the

node develops a left-right asymmetry, which appears to be responsible for the left-right situs of the heart (Levin, M. *et al.* (1995) Cell 82:803-814).

In the CNS, *Shh* from the notochord and the floorplate appears to induce ventral cell fates. When ectopically expressed, *Shh* leads to a ventralization of large regions of the mid- and hindbrain in mouse (Echelard *et al.* (1993) supra; Goodrich, L.V. *et al.* (1996) Genes Dev. 10:301-312), *Xenopus* (Roelink, H. et al. (1994) *supra*; Ruiz i Altaba, A. *et al.* (1995) Mol. Cell. Neurosci. 6:106-121), and zebrafish (Ekker *et al.* (1995) supra; Krauss *et al.* (1993) supra; Hammerschmidt, M., *et al.* (1996) Genes Dev. 10:647-658). In explants of intermediate neuroectoderm at spinal cord levels, *Shh* protein induces floorplate and motor neuron development with distinct concentration thresholds, floor plate at high and motor neurons at lower concentrations (Roelink *et al.* (1995) supra; Marti *et al.* (1995) supra; Tanabe, Y. *et al.* (1995) Curr. Biol. 5:651-658). Moreover, antibody blocking suggests that *Shh* produced by the notochord is required for notochord-mediated induction of motor neuron fates (Marti *et al.* (1995) supra). Thus, high concentrations of *Shh* on the surface of *Shh*-producing midline cells appears to account for the contact-mediated induction of floorplate observed *in vitro* (Placzek, M. *et al.* (1993) Development 117:205-218), and the midline positioning of the floorplate immediately above the notochord *in vivo*. Lower concentrations of *Shh* released from the notochord and the floorplate presumably induce motor neurons at more distant ventrolateral regions in a process that has been shown to be contact-independent *in vitro* (Yamada, T. *et al.* (1993) Cell 73:673-686). In explants taken at midbrain and forebrain levels, *Shh* also induces the appropriate ventrolateral neuronal cell types, dopaminergic (Heynes, M. *et al.* (1995) Neuron 15:35-44; Wang, M.Z. *et al.* (1995) Nature Med. 1:1184-1188) and cholinergic (Ericson, J. *et al.* (1995) Cell 81:747-756) precursors, respectively, indicating that *Shh* is a common inducer of ventral specification over the entire length of the CNS. These observations raise a question as to how the differential response to *Shh* is regulated at particular anteroposterior positions.

Shh from the midline also patterns the paraxial regions of the vertebrate embryo, the somites in the trunk (Fan *et al.* (1995) supra) and the head mesenchyme rostral of the somites (Hammerschmidt *et al.* (1996) supra). In chick and mouse paraxial mesoderm explants, *Shh* promotes the expression of sclerotome specific markers like *Pax1* and *Twist*, at the expense of the dermamyotomal marker *Pax3*. Moreover, filter barrier experiments suggest that *Shh* mediates

the induction of the sclerotome directly rather than by activation of a secondary signaling mechanism (Fan, C.-M. and Tessier-Lavigne, M. (1994) Cell 79, 1175-1186).

Shh also induces myotomal gene expression (Hammerschmidt et al. (1996) supra; Johnson, R.L. *et al.* (1994) Cell 79:1165-1173; Münsterberg, A.E. *et al.* (1995) Genes Dev. 9:2911-2922; Weinberg, E.S. *et al.* (1996) Development 122:271-280), although recent experiments indicate that members of the WNT family, vertebrate homologues of *Drosophila wingless*, are required in concert (Münsterberg *et al.* (1995) supra). Puzzlingly, myotomal induction in chicks requires higher *Shh* concentrations than the induction of sclerotomal markers (Münsterberg *et al.* (1995) supra), although the sclerotome originates from somitic cells positioned much closer to the notochord. Similar results were obtained in the zebrafish, where high concentrations of *Hedgehog* induce myotomal and repress sclerotomal marker gene expression (Hammerschmidt *et al.* (1996) supra). In contrast to amniotes, however, these observations are consistent with the architecture of the fish embryo, as here, the myotome is the predominant and more axial component of the somites. Thus, modulation of *Shh* signaling and the acquisition of new signaling factors may have modified the somite structure during vertebrate evolution.

In the vertebrate limb buds, a subset of posterior mesenchymal cells, the "Zone of polarizing activity" (ZPA), regulates anteroposterior digit identity (reviewed in Honig, L.S. (1981) Nature 291:72-73). Ectopic expression of *Shh* or application of beads soaked in *Shh* peptide mimics the effect of anterior ZPA grafts, generating a mirror image duplication of digits (Chang *et al.* (1994) *supra*; Lopez-Martinez *et al.* (1995) *supra*; Riddle *et al.* (1993) *supra*) (Fig. 2g). Thus, digit identity appears to depend primarily on *Shh* concentration, although it is possible that other signals may relay this information over the substantial distances that appear to be required for AP patterning (100-150 μm). Similar to the interaction of HH and DPP in the *Drosophila* imaginal discs, *Shh* in the vertebrate limb bud activates the expression of *Bmp2* (Francis, P.H. *et al.* (1994) Development 120:209-218), a *dpp* homologue. However, unlike DPP in *Drosophila*, *Bmp2* fails to mimic the polarizing effect of *Shh* upon ectopic application in the chick limb bud (Francis *et al.* (1994) supra). In addition to anteroposterior patterning, *Shh* also appears to be involved in the regulation of the proximodistal outgrowth of the limbs by inducing

the synthesis of the fibroblast growth factor FGF4 in the posterior apical ectodermal ridge (Laufer, E. *et al.* (1994) Cell 79:993-1003; Niswander, L. *et al.* (1994) Nature 371:609-612).

The close relationship between *Hedgehog* proteins and BMPs is likely to have been conserved at many, but probably not all sites of vertebrate *Hedgehog* expression. For example, in the chick hindgut, *Shh* has been shown to induce the expression of *Bmp4*, another vertebrate *dpp* homologue (Roberts, D.J. *et al.* (1995) Development 121:3163-3174). Furthermore, *Shh* and *Bmp2, 4*, or *6* show a striking correlation in their expression in epithelial and mesenchymal cells of the stomach, the urogenital system, the lung, the tooth buds and the hair follicles (Bitgood, M.J. and McMahon, A.P. (1995) Dev. Biol. 172:126-138). Further, *Ihh*, one of the two other mouse *Hedgehog* genes, is expressed adjacent to *Bmp* expressing cells in the gut and developing cartilage (Bitgood and McMahon (1995) *supra*).

Recent evidence suggests a model in which *Ihh* plays a crucial role in the regulation of chondrogenic development (Roberts *et al.* (1995) supra). During cartilage formation, chondrocytes proceed from a proliferating state via an intermediate, prehypertrophic state to differentiated hypertrophic chondrocytes. *Ihh* is expressed in the prehypertrophic chondrocytes and initiates a signaling cascade that leads to the blockage of chondrocyte differentiation. Its direct target is the perichondrium around the *Ihh* expression domain, which responds by the expression of *Gli* and *Patched* (*Ptc*), conserved transcriptional targets of *Hedgehog* signals (see below). Most likely, this leads to secondary signaling resulting in the synthesis of parathyroid hormone-related protein (PTHrP) in the periarticular perichondrium. PTHrP itself signals back to the prehypertrophic chondrocytes, blocking their further differentiation. At the same time, PTHrP represses expression of *Ihh*, thereby forming a negative feedback loop that modulates the rate of chondrocyte differentiation.

Patched was originally identified in Drosophila as a segment polarity gene, one of a group of developmental genes that affect cell differentiation within the individual segments that occur in a homologous series along the anterior-posterior axis of the embryo. See Hooper, J.E. et al. (1989) Cell 59:751; and Nakano, Y. et al. (1989) Nature 341:508. Patterns of expression of the vertebrate homologue of *patched* suggest its involvement in the development of neural tube, skeleton, limbs, craniofacial structure, and skin.

Genetic and functional studies demonstrate that *patched* is part of the *hedgehog* signaling cascade, an evolutionarily conserved pathway that regulates expression of a number of downstream genes. See Perrimon, N. (1995) Cell 80:517; and Perrimon, N. (1996) Cell 86:513. *Patched* participates in the constitutive transcriptional repression of the target genes; its effect is opposed by a secreted glycoprotein, encoded by *hedgehog*, or a vertebrate homologue, which induces transcriptional activation. Genes under control of this pathway include members of the Wnt and TGF-beta families.

Patched proteins possess two large extracellular domains, twelve transmembrane segments, and several cytoplasmic segments. See Hooper, supra; Nakano, supra; Johnson, R.L. et al. (1996) Science 272:1668; and Hahn, H. et al. (1996) Cell 85:841. The biochemical role of *patched* in the *hedgehog* signaling pathway is unclear. Direct interaction with the *hedgehog* protein has, however, been reported (Chen, Y. et al. (1996) Cell 87:553), and *patched* may participate in a *hedgehog* receptor complex along with another transmembrane protein encoded by the *smoothened* gene. See Perrimon, supra; and Chen, supra.

The human homologue of *patched* was recently cloned and mapped to chromosome 9q22.3. See Johnson, supra; and Hahn, supra. This region has been implicated in basal cell nevus syndrome (BCNS), which is characterized by developmental abnormalities including rib and craniofacial alterations, abnormalities of the hands and feet, and spina bifida.

Sporadic tumors also demonstrated a loss of both functional alleles of *patched*. Of twelve tumors in which *patched* mutations were identified with a single strand conformational polymorphism screening assay, nine had chromosomal deletion of the second allele and the other three had inactivating mutations in both alleles (Gailani, supra). The alterations did not occur in the corresponding germline DNA.

Most of the identified mutations resulted in premature stop codons or frame shifts (Lench, N.J., et al., *Hum. Genet.* **1997** Oct; 100(5-6): 497-502). Several, however, were point mutations leading to amino acid substitutions in either extracellular or cytoplasmic domains. These sites of mutation may indicate functional importance for interaction with extracellular proteins or with cytoplasmic members of the downstream signaling pathway.

The involvement of *patched* in the inhibition of gene expression and the occurrence of frequent allelic deletions of *patched* in BCC support a tumor suppressor function for this gene.

Its role in the regulation of gene families known to be involved in cell signaling and intercellular communication provides a possible mechanism of tumor suppression.

Summary of the Invention

In certain aspects, the present invention makes available methods and reagents for inhibiting undesirable growth states that occur in cells with an active *hedgehog* signaling pathway. In one embodiment, the subject methods may be used to inhibit unwanted cell proliferation by determining whether cells overexpress a *gli* gene, and contacting cells that overexpress a *gli* gene with an effective amount of a *hedgehog* antagonist. In preferred embodiments, the unwanted cell proliferation is cancer or benign prostatic hyperplasia.

Another aspect of the present invention makes available methods for determining a treatment protocol comprising obtaining a tissue sample from a patient, and determining levels of *gli* gene expression in said sample, wherein overexpression of a *gli* gene indicates that treatment with a hedgehog antagonist is appropriate.

A further aspect of the invention provides methods for stimulating surfactant production in a lung cell comprising contacting said cell with an amount of *hedgehog* antagonist effective to stimulate surfactant production. Another aspect of the invention provides methods for stimulating lamellated body formation in a lung cell comprising contacting said cell with an amount of *hedgehog* antagonist effective to stimulate lamellated body formation. In preferred embodiments, the lung cell is present in the lung tissue of a premature infant.

In other preferred embodiments, *hedgehog* antagonists of the invention are selected from a small molecule of less than 2000 daltons, a *hedgehog* antibody, a *patched* antibody, a *smoothened* antibody, a mutant *hedgehog* protein, an antisense nucleic acid, and a ribozyme. In particularly preferred embodiments, the *hedgehog* antagonist is selected from one of formulae I through XXV. In particularly preferred embodiments the *hedgehog* antagonist is selected from cyclopamine, compound A, tomatidine, jervine, AY9944, triparanol, compound B, and functionally effective derivatives thereof.

In another aspect, the invention provides methods of determining the likelihood that a cancer will develop in a tissue, comprising obtaining a tissue sample, and determining levels of *gli* gene expression in said sample, wherein, overexpression of a *gli* gene indicates that cancer is more likely to develop.

Brief Description of the Drawings

Figure 1 depicts the chemical structures for AY9944, triparanol, jervine, cyclopamine, tomatidine and cholesterol.

Figure 2A depicts the chemical structure for compound A.

Figure 2B shows the chemical structure for compound B.

Figure 3 shows the chemical structure for agonist Z.

Figure 4 depicts *gli-1* gene expression in embryonic and adult mouse lung.

Figure 5 shows the inverse relationship between *gli-1* expression and the expression of markers of lung maturation. Between E13.5 and E16.5, the expression of *gli-1* decreases while the expression of the maturation marker, surfactant type C (Sp-C), increases.

Figure 6 shows the effect of compound B treatment of embryonic mouse lungs on *gli-1* expression.

Figure 7 shows compound B treatment increases surfactant type C production in embryonic mouse lungs.

Figure 8 shows that type II pneumocytes in compound B-treated lungs differentiate prematurely, as evidenced by the presence of surfactant producing lamellated bodies.

Figure 9 shows that treatment of embryonic lung cultures with compound B decreases expression of *gli-1*.

Figure 10 shows that treatment of embryonic lung cultures with compound B increases expression of the maturation marker Sp-C. The induction of Sp-C observed following treatment

is comparable to that observed following treatment with known lung maturation factor hydrocortisone.

Figure 11 shows that treatment of embryonic lung cultures with hedgehog agonists has the opposite effect. Treatment with either sonic hedgehog or with agonist Z increases *gli-1* expression and decreases Sp-C expression.

Figure 12 illustrates *gli*-1 expression in breast cancer tissue as visualized by in situ hybridization.

Figure 13 shows *gli*-1 expression in lung cancer visualized by in situ hybridization

Figure 14 illustrates *gli*-1 expression in prostate cancer as visualized by in situ hybridization

Figure 15 depicts *gli*-1 expression in benign prostatic hyperplasia as visualized by in situ hybridization

Figure 16 shows: (A). *Ptc-lacZ* transgene expression in newborn mouse *ptc-1 (d11) lacZ* bladder epithelium. *LacZ* expression can be detected in the proliferating urothelial cells and, more weakly, in adjacent mesenchymal cells. (B). *Gli-1* expression in adult mouse bladder epithelium. *Gli-1* expression can be detected in the proliferating urothelial cells.

Figure 17 shows the expression of *gli-1* and *shh* in normal adult bladder and in a commercially available bladder tumor.

Figure 18 shows the expression of *shh* and *gli-1* in eight commercially available bladder cancer cell lines. All eight cell lines examined express genes involved in *hedgehog* signaling.

Figure 19 shows the expression of *shh, ptc-1, smo, gli-1, gli-2, and gli-3* in eight commercially available bladder cancer cell lines, as well as in fetal brain.

Figure 20 shows a schematic representation of the gli-Luc assay.

Figure 21 shows the results of the gli-Luc assay on bladder cancer cell co-cultures. Co-culture of S12 cells with either cell line 5637 or cell line RT4 results in activation of the reporter gene indicating that these cell lines can activate *hedgehog* signaling.

Figure 22 shows that the Shh antibody 5E1 inhibits activation of the reporter gene in RT-4/S12 co-cultures.

Figure 23 and 24 show that administration of the Shh antibody 5E1 inhibits tumor growth in vivo in a nude mouse bladder cancer model.

Figure 25 shows that administration of the Shh antibody 5E1 decreases expression of *gli-1* in vivo in a nude mouse bladder cancer model.

Figure 26 shows that shh is expressed in prostate cancer samples as visualized by in situ hybridization.

Figure 27 shows by Q-RT-PCR the expression of *gli-1* in normal adult prostate and in a prostate adenocarcinoma.

Figure 28 shows the expression of *shh* and *gli-1* in three prostate cancer cell lines in comparison with expression in a normal prostate cell line.

Figure 29 shows that prostate cancer cell lines induce expression of luciferase when co-cultured with S12 cells in the gli-Luc in vitro assay.

Figure 30 shows that the antagonizing antibody 5E1 inhibits the induction of luciferase in by prostate cancer cells in the gli-Luc in vitro assay.

Figure 31 shows the expression of *shh* in prostatic epithelium and stroma in human BPH samples.

Figure 32 shows the expression of *gli-1* in the prostatic stroma of human BPH samples as measured by radioactive in situ hybridization.

Figure 33 shows that *shh* and *patched-1* are expressed in a proximo-distal pattern in normal prostate tissue with the highest levels of gene expression occurring in the proximo or central region.

Figure 34 shows the expression of *shh* and *gli-1* in BPH samples, and compares the levels of gene expression to BCC samples.

Figure 35 shows the expression of *shh* and *gli-1* in BPH cell lines, and compares the levels of gene expression to that of BCC samples, normal prostate, and prostate cancer.

Figure 36 shows that the Shh blocking antibody 5E1 decreases tumor size when administered to mice injected with the Shh expressing colon cancer cell line HT-29.

Figure 37 shows that administration of the Shh blocking antibody 5E1 after 10 days of tumor growth decreases the rate of tumor growth and tumor size in mice injected with the Shh expressing colon cancer cell line HT-29.

Detailed Description of the Invention

I. Overview

The present invention relates to the discovery that signal transduction pathways regulated by *hedgehog, patched* (*ptc*), *gli* and/or *smoothened* can be inhibited, at least in part, by *hedgehog* antagonists. While not wishing to be bound by any theory, in the case of small molecule antagonists, the modulation of a receptor may be the mechanism by which these agents act. For example, the ability of these agents to inhibit proliferation of *patched* loss-of-function (ptc^{lof}) cells may be due to the ability of such molecules to interact with *hedgehog*, *patched*, or *smoothened*, or at least to interfere with the ability of those proteins to activate a *hedgehog*, *ptc*, and/or *smoothened*-mediated signal transduction pathway.

It is, therefore, specifically contemplated that these small molecules which interfere with aspects of *hedgehog*, *ptc*, or *smoothened* signal transduction activity will likewise be capable of changing the role of a cell in tissue development from what would otherwise occur. In preferred embodiments, the cell has a substantially wild-type *hedgehog* signaling pathway. It is also contemplated that *hedgehog* antagonists are particularly effective in treating disorders resulting from hyperactivation of the *hedgehog* pathway as a result of mutations. Therefore, it is desirable to have a method for identifying those cells in which the *hedgehog* pathway is hyperactive such that antagonist treatment may be efficiently targeted.

In certain embodiments, the subject antagonists are organic molecules having a molecular weight less than 2500 amu, more preferably less than 1500 amu, and even more preferably less

than 750 amu, and are capable of inhibiting at least some of the biological activities of *hedgehog* proteins, preferably specifically in target cells.

Thus, the methods of the present invention include the use of small molecules that agonize *ptc* inhibition of *hedgehog* signaling in the regulation of repair and/or functional performance of a wide range of cells, tissues and organs having the phenotype of *hedgehog* gain-of-function and in tissues with wild-type *hedgehog* activity. For instance, the subject method has therapeutic and cosmetic applications ranging from regulation of neural tissues, bone and cartilage formation and repair, regulation of spermatogenesis, regulation of smooth muscle, regulation of lung, liver and tissue of other organs arising from the primitive gut, regulation of hematopoietic function, regulation of skin and hair growth, etc. Moreover, the subject methods can be performed on cells that are provided in culture (*in vitro*), or on cells in a whole animal (*in vivo*). See, for example, PCT publications WO 95/18856 and WO 96/17924 (the specifications of which are expressly incorporated by reference herein).

In another aspect, the present invention provides pharmaceutical preparations comprising, as an active ingredient, a *hedgehog* antagonist or *ptc* agonist such as described herein, formulated in an amount sufficient to inhibit, *in vivo*, proliferation or other biological consequences of *hedgehog* gain-of-function.

The subject treatments using *hedgehog* antagonists can be effective for both human and animal subjects. Animal subjects to which the invention is applicable extend to both domestic animals and livestock, raised either as pets or for commercial purposes. Examples are dogs, cats, cattle, horses, sheep, hogs, and goats.

II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

The phrase "aberrant modification or mutation" of a gene refers to such genetic lesions as, for example, deletions, substitution or addition of nucleotides to a gene, as well as gross chromosomal rearrangements of the gene and/or abnormal methylation of the gene. Likewise, misexpression of a gene refers to aberrant levels of transcription of the gene relative to those

levels in a normal cell under similar conditions, as well as non-wild-type splicing of mRNA transcribed from the gene.

The term "adenocarcinoma" as used herein refers to a malignant tumor originating in glandular epithelium.

The term "angiogenesis", as used herein, refers to the formation of blood vessels. Specifically, angiogenesis is a multistep process in which endothelial cells focally degrade and invade through their own basement membrane, migrate through interstitial stroma toward an angiogenic stimulus, proliferate proximal to the migrating tip, organize into blood vessels, and reattach to newly synthesized basement membrane (see Folkman et al., Adv. Cancer Res., Vol. 43, pp. 175-203 (1985)).

"Basal cell carcinomas" exist in a variety of clinical and histological forms such as nodular-ulcerative, superficial, pigmented, morphealike, fibroepithelioma and nevoid syndrome. Basal cell carcinomas are the most common cutaneous neoplasms found in humans. The majority of new cases of nonmelanoma skin cancers fall into this category.

"Benign prostatic hyperplasia", or BPH, is a benign enlargement of the prostate gland that begins normally after age 50 years probably secondary to the effects of male hormones. If significant enlargement occurs, it may pinch off the urethra making urination difficult or impossible.

"Burn wounds" refer to cases where large surface areas of skin have been removed or lost from an individual due to heat and/or chemical agents.

The term "carcinoma" refers to a malignant new growth made up of epithelial cells tending to infiltrate surrounding tissues and to give rise to metastases. Exemplary carcinomas include: "basal cell carcinoma", which is an epithelial tumor of the skin that, while seldom metastasizing, has potentialities for local invasion and destruction; "squamous cell carcinoma", which refers to carcinomas arising from squamous epithelium and having cuboid cells; "carcinosarcoma", which include malignant tumors composed of carcinomatous and sarcomatous tissues; "adenocystic carcinoma", carcinoma marked by cylinders or bands of hyaline or mucinous stroma separated or surrounded by nests or cords of small epithelial cells, occurring in the mammary and salivary glands, and mucous glands of the respiratory tract; "epidermoid

carcinoma", which refers to cancerous cells which tend to differentiate in the same way as those of the epidermis; i.e., they tend to form prickle cells and undergo cornification; "nasopharyngeal carcinoma", which refers to a malignant tumor arising in the epithelial lining of the space behind the nose; and "renal cell carcinoma", which pertains to carcinoma of the renal parenchyma composed of tubular cells in varying arrangements. Other carcinomatous epithelial growths are "papillomas", which refers to benign tumors derived from epithelium and having a papillomavirus as a causative agent; and "epidermoidomas", which refers to a cerebral or meningeal tumor formed by inclusion of ectodermal elements at the time of closure of the neural groove.

The "corium" or "dermis" refers to the layer of the skin deep to the epidermis, consisting of a dense bed of vascular connective tissue, and containing the nerves and terminal organs of sensation. The hair roots, and sebaceous and sweat glands are structures of the epidermis which are deeply embedded in the dermis.

"Dental tissue" refers to tissue in the mouth that is similar to epithelial tissue, for example gum tissue. The method of the present invention is useful for treating periodontal disease.

"Dermal skin ulcers" refer to lesions on the skin caused by superficial loss of tissue, usually with inflammation. Dermal skin ulcers that can be treated by the method of the present invention include decubitus ulcers, diabetic ulcers, venous stasis ulcers and arterial ulcers. Decubitus wounds refer to chronic ulcers that result from pressure applied to areas of the skin for extended periods of time. Wounds of this type are often called bedsores or pressure sores. Venous stasis ulcers result from the stagnation of blood or other fluids from defective veins. Arterial ulcers refer to necrotic skin in the area around arteries having poor blood flow.

The term "ED_{50}" means the dose of a drug that produces 50% of its maximum response or effect.

An "effective amount" of, e.g., a *hedgehog* antagonist, with respect to the subject method of treatment, refers to an amount of the antagonist in a preparation which, when applied as part of a desired dosage regimen brings about, e.g., a change in the rate of cell proliferation and/or the state of differentiation of a cell and/or rate of survival of a cell according to clinically acceptable standards for the disorder to be treated or for the cosmetic purpose.

The terms "epithelia", "epithelial" and "epithelium" refer to the cellular covering of internal and external body surfaces (cutaneous, mucous and serous), including the glands and other structures derived therefrom, e.g., corneal, esophegeal, epidermal, and hair follicle epithelial cells. Other exemplary epithelial tissue includes: olfactory epithelium, which is the pseudostratified epithelium lining the olfactory region of the nasal cavity, and containing the receptors for the sense of smell; glandular epithelium, which refers to epithelium composed of secreting cells; squamous epithelium, which refers to epithelium composed of flattened plate-like cells. The term epithelium can also refer to transitional epithelium, like that which is characteristically found lining hollow organs that are subject to great mechanical change due to contraction and distention, e.g., tissue which represents a transition between stratified squamous and columnar epithelium.

The term "epithelialization" refers to healing by the growth of epithelial tissue over a denuded surface.

The term "epidermal gland" refers to an aggregation of cells associated with the epidermis and specialized to secrete or excrete materials not related to their ordinary metabolic needs. For example, "sebaceous glands" are holocrine glands in the corium that secrete an oily substance and sebum. The term "sweat glands" refers to glands that secrete sweat, situated in the corium or subcutaneous tissue, opening by a duct on the body surface.

The term "epidermis" refers to the outermost and nonvascular layer of the skin, derived from the embryonic ectoderm, varying in thickness from 0.07-1.4 mm. On the palmar and plantar surfaces it comprises, from within outward, five layers: basal layer composed of columnar cells arranged perpendicularly; prickle-cell or spinous layer composed of flattened polyhedral cells with short processes or spines; granular layer composed of flattened granular cells; clear layer composed of several layers of clear, transparent cells in which the nuclei are indistinct or absent; and horny layer composed of flattened, cornified non-nucleated cells. In the epidermis of the general body surface, the clear layer is usually absent.

"Excisional wounds" include tears, abrasions, cuts, punctures or lacerations in the epithelial layer of the skin and may extend into the dermal layer and even into subcutaneous fat and beyond. Excisional wounds can result from surgical procedures or from accidental penetration of the skin.

The "growth state" of a cell refers to the rate of proliferation of the cell and/or the state of differentiation of the cell. An "altered growth state" is a growth state characterized by an abnormal rate of proliferation, e.g., a cell exhibiting an increased or decreased rate of proliferation relative to a normal cell.

The term "hair" refers to a threadlike structure, especially the specialized epidermal structure composed of keratin and developing from a papilla sunk in the corium, produced only by mammals and characteristic of that group of animals. Also, "hair" may refer to the aggregate of such hairs. A "hair follicle" refers to one of the tubular-invaginations of the epidermis enclosing the hairs, and from which the hairs grow. "Hair follicle epithelial cells" refers to epithelial cells that surround the dermal papilla in the hair follicle, e.g., stem cells, outer root sheath cells, matrix cells, and inner root sheath cells. Such cells may be normal non-malignant cells, or transformed/immortalized cells.

The term "*hedgehog*" is used to refer generically to any member of the *hedgehog* family, including sonic, indian, desert and tiggy winkle. The term may be used to indicate protein or gene.

The term "*hedgehog* signaling pathway", "*hedgehog* pathway" and "*hedgehog* signal transduction pathway" are all used to refer to the chain of events normally mediated by *hedgehog*, *smoothened*, *ptc*, and *gli*, among others, and resulting in a changes in gene expression and other phenotypic changes typical of *hedgehog* activity. The *hedgehog* pathway can be activated even in the absence of a *hedgehog* protein by activating a downstream component. For example, overexpression of *smoothened* will activate the pathway in the absence of *hedgehog*. *gli* and *ptc* gene expression are indicators of an active *hedgehog* signaling pathway.

The term "*hedgehog* antagonist" refers to an agent that potentiates or recapitulates the bioactivity of *patched*, such as to repress transcription of target genes. Preferred *hedgehog* antagonists can be used to overcome a *ptc* loss-of-function and/or a *smoothened* gain-of-function, the latter also being referred to as *smoothened* antagonists. The term '*hedgehog* antagonist' as used herein refers not only to any agent that may act by directly inhibiting the normal function of the *hedgehog* protein, but also to any agent that inhibits the *hedgehog* signalling pathway, and thus recapitulates the function of *ptc*. A *hedgehog* antagonist may be a small molecule, an antibody (including but not restricted to: a diabody, single chain antibody,

monoclonal antibody, IgG, IgM, IgA, IgD, IgE, or an antibody fragment comprising at least one pair of variable regions), an antisense nucleic acid, PNA or ribozyme, or a mutant *hedgehog* protein that can disrupt or inhibit *hedgehog* signaling. An antibody may be directed to a protein encoded by any of the genes in the *hedgehog* pathway, including *sonic*, *indian* or *desert hedgehog*, *smoothened*, *ptc-1*, *ptc-2*, *gli-1*, *gli-2*, *gli-3*, etc. In most cases, the antibody would inhibit the activity of the target protein, but in the case of *patched*, such an antibody would be an activator of *patched*. An antisense nucleic acid would likewise decrease production of a protein encoded by any of the genes in the *hedgehog* pathway, with the exception of *patched* or other genes encoding negative regulators of the *hedgehog* signaling pathway.

The term "*hedgehog* gain-of-function" refers to an aberrant modification or mutation of a *ptc* gene, *hedgehog* gene, or *smoothened* gene, or a decrease (or loss) in the level of expression of such a gene, which results in a phenotype which resembles contacting a cell with a *hedgehog* protein, e.g., aberrant activation of a *hedgehog* pathway. The gain-of-function may include a loss of the ability of the *ptc* gene product to regulate the level of expression of Ci genes, e.g., *Gli1*, *Gli2*, and *Gli3*. The term '*hedgehog* gain-of-function' is also used herein to refer to any similar cellular phenotype (e.g., exhibiting excess proliferation) that occurs due to an alteration anywhere in the *hedgehog* signal transduction pathway, including, but not limited to, a modification or mutation of *hedgehog* itself. For example, a tumor cell with an abnormally high proliferation rate due to activation of the *hedgehog* signalling pathway would have a '*hedgehog* gain-of-function' phenotype, even if *hedgehog* is not mutated in that cell.

As used herein, "immortalized cells" refers to cells that have been altered via chemical and/or recombinant means such that the cells have the ability to grow through an indefinite number of divisions in culture.

"Internal epithelial tissue" refers to tissue inside the body that has characteristics similar to the epidermal layer in the skin. Examples include the lining of the intestine. The method of the present invention is useful for promoting the healing of certain internal wounds, for example wounds resulting from surgery.

The term "keratosis" refers to proliferative skin disorder characterized by hyperplasia of the horny layer of the epidermis. Exemplary keratotic disorders include keratosis follicularis, keratosis palmaris et plantaris, keratosis pharyngea, keratosis pilaris, and actinic keratosis.

“Lamellated bodies” refers to a subcellular structure found in lung cells that are producing surfactants. Lamellated bodies are thought to be the site of lung surfactant biosynthesis. The bodies have a multilayered membranous appearance in an electron micrograph.

The term “LD_{50}” means the dose of a drug that is lethal in 50% of test subjects.

The term "nail" refers to the horny cutaneous plate on the dorsal surface of the distal end of a finger or toe.

The term “overexpression” as used in reference to gene expression levels means any level of gene expression in cells of a tissue that is higher than the normal level of expression for that tissue. The normal level of expression for a tissue may be assessed by measuring gene expression in a healthy portion of that tissue.

The term “*patched* loss-of-function” refers to an aberrant modification or mutation of a *ptc* gene, or a decreased level of expression of the gene, which results in a phenotype that resembles contacting a cell with a *hedgehog* protein, e.g., aberrant activation of a *hedgehog* pathway. The loss-of-function may include a loss of the ability of the *ptc* gene product to regulate the level of expression of Ci genes, e.g., *Gli1*, *Gli2* and *Gli3*.

A "patient" or "subject" to be treated by the subject method can mean either a human or non-human animal.

The term “prodrug” is intended to encompass compounds that, under physiological conditions, are converted into the therapeutically active agents of the present invention. A common method for making a prodrug is to include selected moieties that are hydrolyzed under physiological conditions to reveal the desired molecule. In other embodiments, the prodrug is converted by an enzymatic activity of the host animal.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

Throughout this application, the term "proliferative skin disorder" refers to any disease/disorder of the skin marked by unwanted or aberrant proliferation of cutaneous tissue. These conditions are typically characterized by epidermal cell proliferation or incomplete cell differentiation, and include, for example, X-linked ichthyosis, psoriasis, atopic dermatitis, allergic contact dermatitis, epidermolytic hyperkeratosis, and seborrheic dermatitis. For example, epidermodysplasia is a form of faulty development of the epidermis. Another example is

"epidermolysis", which refers to a loosened state of the epidermis with formation of blebs and bullae either spontaneously or at the site of trauma.

As used herein, the term "psoriasis" refers to a hyperproliferative skin disorder that alters the skin's regulatory mechanisms. In particular, lesions are formed which involve primary and secondary alterations in epidermal proliferation, inflammatory responses of the skin, and an expression of regulatory molecules such as lymphokines and inflammatory factors. Psoriatic skin is morphologically characterized by an increased turnover of epidermal cells, thickened epidermis, abnormal keratinization, inflammatory cell infiltrates into the dermis layer and polymorphonuclear leukocyte infiltration into the epidermis layer resulting in an increase in the basal cell cycle. Additionally, hyperkeratotic and parakeratotic cells are present.

The term "skin" refers to the outer protective covering of the body, consisting of the corium and the epidermis, and is understood to include sweat and sebaceous glands, as well as hair follicle structures. Throughout the present application, the adjective "cutaneous" may be used, and should be understood to refer generally to attributes of the skin, as appropriate to the context in which they are used.

The term "small cell carcinoma" refers to a type of malignant neoplasm, commonly of the bronchus. Cells of the tumor have endocrine like characteristics and may secrete one or more of a wide range of hormones, especially regulatory peptides like bombesin.

The term "*smoothened* gain-of-function" refers to an aberrant modification or mutation of a *smo* gene, or an increased level of expression of the gene, which results in a phenotype that resembles contacting a cell with a *hedgehog* protein, e.g., aberrant activation of a *hedgehog* pathway. While not wishing to be bound by any particular theory, it is noted that *ptc* may not signal directly into the cell, but rather interact with *smoothened*, another membrane-bound protein located downstream of *ptc* in *hedgehog* signaling (Marigo et al., (1996) Nature 384: 177-179). The gene *smo* is a segment-polarity gene required for the correct patterning of every segment in Drosophila (Alcedo et al., (1996) Cell 86: 221-232). Human homologs of *smo* have been identified. See, for example, Stone et al. (1996) Nature 384:129-134, and GenBank accession U84401. The *smoothened* gene encodes an integral membrane protein with characteristics of heterotrimeric G-protein-coupled receptors; i.e., 7-transmembrane regions. This protein shows homology to the Drosophila *Frizzled* (Fz) protein, a member of the *wingless*

pathway. It was originally thought that *smo* encodes a receptor of the Hh signal. However, this suggestion was subsequently disproved, as evidence for *ptc* being the Hh receptor was obtained. Cells that express *Smo* fail to bind Hh, indicating that *smo* does not interact directly with Hh (Nusse, (1996) Nature 384: 119-120). Rather, the binding of *Sonic hedgehog* (SHH) to its receptor, *PTC*H, is thought to prevent normal inhibition by *PTC*H of *smoothened* (SMO), a seven-span transmembrane protein.

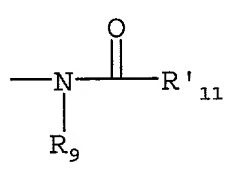
Recently, it has been reported that activating *smoothened* mutations occur in sporadic basal cell carcinoma, Xie et al. (1998) Nature 391: 90-2, and primitive neuroectodermal tumors of the central nervous system, Reifenberger et al. (1998) Cancer Res 58: 1798-803.

The term "therapeutic index" refers to the therapeutic index of a drug defined as LD_{50}/ED_{50}.

As used herein, "transformed cells" refers to cells that have spontaneously converted to a state of unrestrained growth, i.e., they have acquired the ability to grow through an indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with respect to their loss of growth control.

"Urogenital" refers to the organs and tissues of the urogenital tract, which includes among other tissues, the prostate, ureter, kidney and bladder. A "urogenital cancer" is a cancer of a urogenital tissue.

The term "acylamino" is art-recognized and refers to a moiety that can be represented by the general formula:



wherein R_9 is as defined above, and R'_{11} represents a hydrogen, an alkyl, an alkenyl or $-(CH_2)_m-R_8$, where m and R_8 are as defined above.

Herein, the term "aliphatic group" refers to a straight-chain, branched-chain, or cyclic aliphatic hydrocarbon group and includes saturated and unsaturated aliphatic groups, such as an alkyl group, an alkenyl group, and an alkynyl group.

The terms "alkenyl" and "alkynyl" refer to unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively.

The terms "alkoxyl" or "alkoxy" as used herein refers to an alkyl group, as defined above, having an oxygen radical attached thereto. Representative alkoxyl groups include methoxy, ethoxy, propyloxy, tert-butoxy and the like. An "ether" is two hydrocarbons covalently linked by an oxygen. Accordingly, the substituent of an alkyl that renders that alkyl an ether is or resembles an alkoxyl, such as can be represented by one of -O-alkyl, -O-alkenyl, -O-alkynyl, -O-$(CH_2)_m$-R_8, where m and R_8 are described above.

The term "alkyl" refers to the radical of saturated aliphatic groups, including straight-chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl-substituted cycloalkyl groups, and cycloalkyl-substituted alkyl groups. In preferred embodiments, a straight chain or branched chain alkyl has 30 or fewer carbon atoms in its backbone (e.g., C_1-C_{30} for straight chains, C_3-C_{30} for branched chains), and more preferably 20 or fewer. Likewise, preferred cycloalkyls have from 3-10 carbon atoms in their ring structure, and more preferably have 5, 6 or 7 carbons in the ring structure.

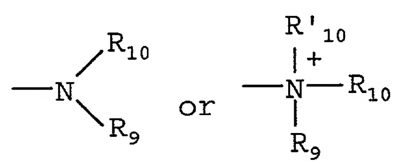
Moreover, the term "alkyl" (or "lower alkyl") as used throughout the specification, examples, and claims is intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, a halogen, a hydroxyl, a carbonyl (such as a carboxyl, an alkoxycarbonyl, a formyl, or an acyl), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an alkoxyl, a phosphoryl, a phosphate, a phosphonate, a phosphinate, an amino, an amido, an amidine, an imine, a cyano, a nitro, an azido, a sulfhydryl, an alkylthio, a sulfate, a sulfonate, a sulfamoyl, a sulfonamido, a sulfonyl, a heterocyclyl, an aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may

include substituted and unsubstituted forms of amino, azido, imino, amido, phosphoryl (including phosphonate and phosphinate), sulfonyl (including sulfate, sulfonamido, sulfamoyl and sulfonate), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters), $-CF_3$, -CN and the like. Exemplary substituted alkyls are described below. Cycloalkyls can be further substituted with alkyls, alkenyls, alkoxys, alkylthios, aminoalkyls, carbonyl-substituted alkyls, $-CF_3$, -CN, and the like.

Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, but having from one to ten carbons, more preferably from one to six carbon atoms in its backbone structure. Likewise, "lower alkenyl" and "lower alkynyl" have similar chain lengths. Throughout the application, preferred alkyl groups are lower alkyls. In preferred embodiments, a substituent designated herein as alkyl is a lower alkyl.

The term "alkylthio" refers to an alkyl group, as defined above, having a sulfur radical attached thereto. In preferred embodiments, the "alkylthio" moiety is represented by one of -S-alkyl, -S-alkenyl, -S-alkynyl, and $-S-(CH_2)_m-R_8$, wherein m and R_8 are defined above. Representative alkylthio groups include methylthio, ethylthio, and the like.

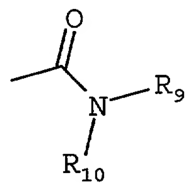
The terms "amine" and "amino" are art-recognized and refer to both unsubstituted and substituted amines, e.g., a moiety that can be represented by the general formula:



wherein R_9, R_{10} and R'_{10} each independently represent a hydrogen, an alkyl, an alkenyl, $-(CH_2)_m-R_8$, or R_9 and R_{10} taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure; R_8 represents an aryl, a cycloalkyl, a cycloalkenyl, a heterocycle or a polycycle; and m is zero or an integer in the range of 1 to 8. In preferred embodiments, only one of R_9 or R_{10} can be a carbonyl, e.g., R_9, R_{10} and the nitrogen together do not form an imide. In even more preferred embodiments, R_9 and R_{10} (and optionally R'_{10}) each independently represent a hydrogen, an alkyl, an alkenyl, or $-(CH_2)_m-R_8$. Thus, the

term "alkylamine" as used herein means an amine group, as defined above, having a substituted or unsubstituted alkyl attached thereto, i.e., at least one of R_9 and R_{10} is an alkyl group.

The term "amido" is art-recognized as an amino-substituted carbonyl and includes a moiety that can be represented by the general formula:



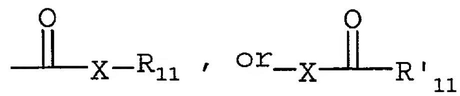
wherein R_9, R_{10} are as defined above. Preferred embodiments of the amide will not include imides, which may be unstable.

The term "aralkyl", as used herein, refers to an alkyl group substituted with an aryl group (e.g., an aromatic or heteroaromatic group).

The term "aryl" as used herein includes 5-, 6-, and 7-membered single-ring aromatic groups that may include from zero to four heteroatoms, for example, benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine and pyrimidine, and the like. Those aryl groups having heteroatoms in the ring structure may also be referred to as "aryl heterocycles" or "heteroaromatics." The aromatic ring can be substituted at one or more ring positions with such substituents as described above, for example, halogen, azide, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, alkoxyl, amino, nitro, sulfhydryl, imino, amido, phosphate, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, sulfonamido, ketone, aldehyde, ester, heterocyclyl, aromatic or heteroaromatic moieties, $-CF_3$, -CN, or the like. The term "aryl" also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings (the rings are "fused rings") wherein at least one of the rings is aromatic, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls.

The term "carbocycle", as used herein, refers to an aromatic or non-aromatic ring in which each atom of the ring is carbon.

The term "carbonyl" is art-recognized and includes such moieties as can be represented by the general formula:



wherein X is a bond or represents an oxygen or a sulfur, and R_{11} represents a hydrogen, an alkyl, an alkenyl, $-(CH_2)_m-R_8$ or a pharmaceutically acceptable salt, R'_{11} represents a hydrogen, an alkyl, an alkenyl or $-(CH_2)_m-R_8$, where m and R_8 are as defined above. Where X is an oxygen and R_{11} or R'_{11} is not hydrogen, the formula represents an "ester". Where X is an oxygen, and R_{11} is as defined above, the moiety is referred to herein as a carboxyl group, and particularly when R_{11} is a hydrogen, the formula represents a "carboxylic acid". Where X is an oxygen, and R'_{11} is hydrogen, the formula represents a "formate". In general, where the oxygen atom of the above formula is replaced by sulfur, the formula represents a "thiocarbonyl" group. Where X is a sulfur and R_{11} or R'_{11} is not hydrogen, the formula represents a "thioester." Where X is a sulfur and R_{11} is hydrogen, the formula represents a "thiocarboxylic acid." Where X is a sulfur and R_{11}' is hydrogen, the formula represents a "thiolformate." On the other hand, where X is a bond, and R_{11} is not hydrogen, the above formula represents a "ketone" group. Where X is a bond, and R_{11} is hydrogen, the above formula represents an "aldehyde" group.

The term "heteroatom" as used herein means an atom of any element other than carbon or hydrogen. Preferred heteroatoms are boron, nitrogen, oxygen, phosphorus, sulfur and selenium.

The terms "heterocyclyl" or "heterocyclic group" refer to 3- to 10-membered ring structures, more preferably 3- to 7-membered rings, whose ring structures include one to four heteroatoms. Heterocycles can also be polycycles. Heterocyclyl groups include, for example, thiophene, thianthrene, furan, pyran, isobenzofuran, chromene, xanthene, phenoxathiin, pyrrole, imidazole, pyrazole, isothiazole, isoxazole, pyridine, pyrazine, pyrimidine, pyridazine, indolizine, isoindole, indole, indazole, purine, quinolizine, isoquinoline, quinoline, phthalazine, naphthyridine, quinoxaline, quinazoline, cinnoline, pteridine, carbazole, carboline, phenanthridine, acridine, pyrimidine, phenanthroline, phenazine, phenarsazine, phenothiazine, furazan, phenoxazine, pyrrolidine, oxolane, thiolane, oxazole, piperidine, piperazine, morpholine, lactones, lactams such as azetidinones and pyrrolidinones, sultams, sultones, and the like. The heterocyclic ring can be substituted at one or more positions with such substituents as

described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphate, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, $-CF_3$, -CN, or the like.

As used herein, the term "nitro" means $-NO_2$; the term "halogen" designates -F, -Cl, -Br or -I; the term "sulfhydryl" means -SH; the term "hydroxyl" means -OH; and the term "sulfonyl" means $-SO_2-$.

The terms "polycyclyl" or "polycyclic group" refer to two or more rings (e.g., cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls) in which two or more carbons are common to two adjoining rings, e.g., the rings are "fused rings". Rings that are joined through non-adjacent atoms are termed "bridged" rings. Each of the rings of the polycycle can be substituted with such substituents as described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphate, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, $-CF_3$, -CN, or the like.

The phrase "protecting group" as used herein means temporary substituents that protect a potentially reactive functional group from undesired chemical transformations. Examples of such protecting groups include esters of carboxylic acids, silyl ethers of alcohols, and acetals and ketals of aldehydes and ketones, respectively. The field of protecting group chemistry has been reviewed (Greene, T.W.; Wuts, P.G.M. *Protective Groups in Organic Synthesis*, 2nd ed.; Wiley: New York, 1991).

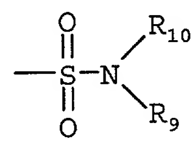
A "selenoalkyl" refers to an alkyl group having a substituted seleno group attached thereto. Exemplary "selenoethers" which may be substituted on the alkyl are selected from one of -Se-alkyl, -Se-alkenyl, -Se-alkynyl, and $-Se-(CH_2)_m-R_8$, m and R_8 being defined above.

As used herein, the term "substituted" is contemplated to include all permissible substituents of organic compounds. In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic substituents of organic compounds. Illustrative substituents include, for example, those described herein above. The permissible substituents can be one or more and the same or

different for appropriate organic compounds. For purposes of this invention, the heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valences of the heteroatoms. This invention is not intended to be limited in any manner by the permissible substituents of organic compounds.

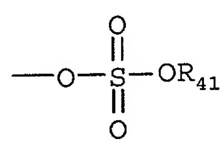
It will be understood that "substitution" or "substituted with" includes the implicit proviso that such substitution is in accordance with permitted valence of the substituted atom and the substituent, and that the substitution results in a stable compound, e.g., which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, etc.

The term "sulfamoyl" is art-recognized and includes a moiety that can be represented by the general formula:



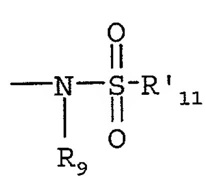
in which R_9 and R_{10} are as defined above.

The term "sulfate" is art recognized and includes a moiety that can be represented by the general formula:



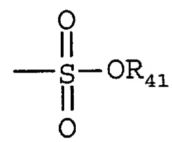
in which R_{41} is as defined above.

The term "sulfonamido" is art recognized and includes a moiety that can be represented by the general formula:



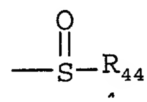
in which R_9 and R'_{11} are as defined above.

The term "sulfonate" is art-recognized and includes a moiety that can be represented by the general formula:



in which R_{41} is an electron pair, hydrogen, alkyl, cycloalkyl, or aryl.

The terms "sulfoxido" or "sulfinyl", as used herein, refers to a moiety that can be represented by the general formula:



in which R_{44} is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aralkyl, or aryl.

Analogous substitutions can be made to alkenyl and alkynyl groups to produce, for example, aminoalkenyls, aminoalkynyls, amidoalkenyls, amidoalkynyls, iminoalkenyls, iminoalkynyls, thioalkenyls, thioalkynyls, carbonyl-substituted alkenyls or alkynyls.

As used herein, the definition of each expression, e.g., alkyl, m, n, etc., when it occurs more than once in any structure, is intended to be independent of its definition elsewhere in the same structure.

The terms triflyl, tosyl, mesyl, and nonaflyl are art-recognized and refer to trifluoromethanesulfonyl, *p*-toluenesulfonyl, methanesulfonyl, and nonafluorobutanesulfonyl groups, respectively. The terms triflate, tosylate, mesylate, and nonaflate are art-recognized and refer to trifluoromethanesulfonate ester, *p*-toluenesulfonate ester, methanesulfonate ester, and nonafluorobutanesulfonate ester functional groups and molecules that contain said groups, respectively.

The abbreviations Me, Et, Ph, Tf, Nf, Ts, Ms represent methyl, ethyl, phenyl, trifluoromethanesulfonyl, nonafluorobutanesulfonyl, *p*-toluenesulfonyl and methanesulfonyl, respectively. A more comprehensive list of the abbreviations utilized by organic chemists of ordinary skill in the art appears in the first issue of each volume of the *Journal of Organic*

Chemistry; this list is typically presented in a table entitled Standard List of Abbreviations. The abbreviations contained in said list, and all abbreviations utilized by organic chemists of ordinary skill in the art are hereby incorporated by reference.

Certain compounds of the present invention may exist in particular geometric or stereoisomeric forms. The present invention contemplates all such compounds, including cis- and trans-isomers, *R*- and *S*-enantiomers, diastereomers, (D)-isomers, (L)-isomers, the racemic mixtures thereof, and other mixtures thereof, as falling within the scope of the invention. Additional asymmetric carbon atoms may be present in a substituent such as an alkyl group. All such isomers, as well as mixtures thereof, are intended to be included in this invention.

If, for instance, a particular enantiomer of a compound of the present invention is desired, it may be prepared by asymmetric synthesis, or by derivation with a chiral auxiliary, where the resulting diastereomeric mixture is separated and the auxiliary group cleaved to provide the pure desired enantiomers. Alternatively, where the molecule contains a basic functional group, such as amino, or an acidic functional group, such as carboxyl, diastereomeric salts may be formed with an appropriate optically active acid or base, followed by resolution of the diastereomers thus formed by fractional crystallization or chromatographic means well known in the art, and subsequent recovery of the pure enantiomers.

Contemplated equivalents of the compounds described above include compounds which otherwise correspond thereto, and which have the same general properties thereof (e.g., the ability to inhibit *hedgehog* signaling), wherein one or more simple variations of substituents are made which do not adversely affect the efficacy of the compound. In general, the compounds of the present invention may be prepared by the methods illustrated in the general reaction schemes as, for example, described below, or by modifications thereof, using readily available starting materials, reagents and conventional synthesis procedures. In these reactions, it is also possible to make use of variants that are in themselves known, but are not mentioned here.

For purposes of this invention, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics, 67th Ed., 1986-87, inside cover. Also for purposes of this invention, the term "hydrocarbon" is contemplated to include all permissible compounds having at least one hydrogen and one carbon atom. In a broad aspect, the permissible hydrocarbons include acyclic and cyclic, branched and

unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic organic compounds which can be substituted or unsubstituted.

III. Exemplary Compounds and Synthesis Thereof

Hedgehog antagonists of the invention may be essentially any composition that inhibits the activity of the *hedgehog* signaling pathway, in other words mimicking the effect of *patched* activity. *Hedgehog* antagonists may be small molecules (organic or inorganic), antisense nucleotides and PNAs, antibodies and altered *hedgehog* proteins.

Small molecule antagonists:

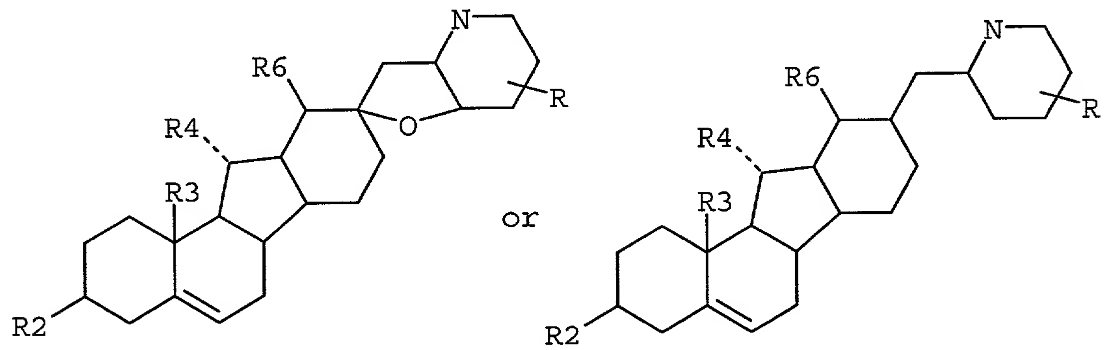
Hedgehog antagonist compounds for certain embodiments of the invention are described in the formulas below and methods of making the compositions are described in detail in the following U.S. Patent applications: 09/663,835, 09/685,244, 09/724,277, 09/687,800, 09/688,018, 60/308,449 and 09/688,076. The exemplary compounds are divided into five parts. For each of the parts, the variable groups and numbers (e.g., R_1, L, Z_2) are individually and distinctly defined, and are internally consistent but not necessarily consistent from part to part.

Exemplary compounds part 1:

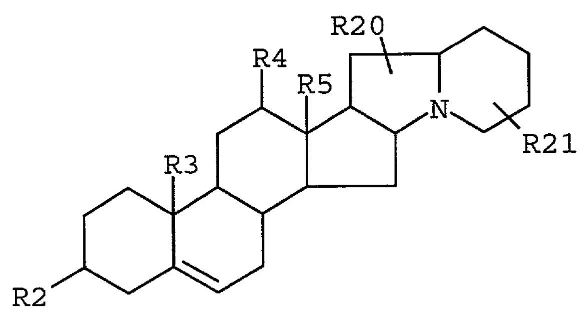
As described in further detail below, it is contemplated that the subject methods can be carried out using any of a variety of different steroidal alkaloids which can be readily identified, e.g., by such drug screening assays as described herein. Steroidal alkaloids have a fairly complex nitrogen-containing nucleus. Two exemplary classes of steroidal alkaloids for use in the subject methods are the Solanum type and the Veratrum type. The above notwithstanding, in a preferred embodiment, the methods and compositions of the present invention make use of compounds having a steroidal alkaloid ring system of cyclopamine.

There are more than 50 naturally occurring veratrum alkaloids including veratramine, cyclopamine, cycloposine, jervine, and muldamine occurring in plants of the Veratrum spp. The Zigadenus spp., death camas, also produces several veratrum-type of steroidal alkaloids including zygacine. In general, many of the veratrum alkaloids (e.g., jervine, cyclopamine and

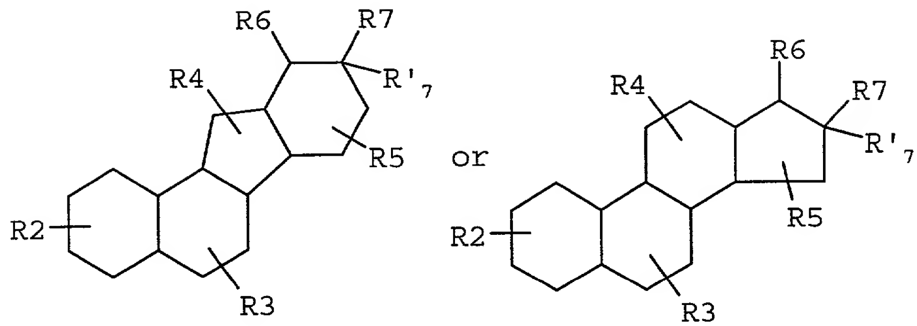
cycloposine) consist of a modified steroid skeleton attached spiro to a furanopiperidine. A typical veratrum-type alkaloid may be represented by:



An example of the Solanum type is solanidine. This steroidal alkaloid is the nucleus (i.e., aglycone) for two important glycoalkaloids, solanine and chaconine, found in potatoes. Other plants in the Solanum family including various nightshades, Jerusalem cherries, and tomatoes also contain solanum-type glycoalkaloids. Glycoalkaloids are glycosides of alkaloids. A typical solanum-type alkaloid may be represented by:



Based on these structures, and the possibility that certain unwanted side effects can be reduced by some manipulation of the structure, a wide range of steroidal alkaloids are contemplated as potential *smoothened* antagonists for use in the subject method. For example, compounds useful in the subject methods include steroidal alkaloids represented in the general formulas (I), or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula I

wherein, as valence and stability permit,

R_2, R_3, R_4, and R_5, represent one or more substitutions to the ring to which each is attached, for each occurrence, independently represent hydrogen, halogens, alkyls, alkenyls, alkynyls, aryls, hydroxyl, =O, =S, alkoxyl, silyloxy, amino, nitro, thiol, amines, imines, amides, phosphoryls, phosphonates, phosphines, carbonyls, carboxyls, carboxamides, anhydrides, silyls, ethers, thioethers, alkylsulfonyls, arylsulfonyls, selenoethers, ketones, aldehydes, esters, sugar (e.g., monosaccharide, disaccharide, polysaccharide, etc.), carbamate (e.g., attached to the steroid at oxygen), carbonate, or $-(CH_2)_m-R_8$;

R_6, R_7, and R'_7, are absent or represent, independently, halogens, alkyls, alkenyls, alkynyls, aryls, hydroxyl, =O, =S, alkoxyl, silyloxy, amino, nitro, thiol, amines, imines, amides, phosphoryls, phosphonates, phosphines, carbonyls, carboxyls, carboxamides, anhydrides, silyls, ethers, thioethers, alkylsulfonyls, arylsulfonyls, selenoethers, ketones, aldehydes, esters, or $-(CH_2)_m-R_8$, or

R_6 and R_7, or R_7 and R'_7, taken together form a ring or polycyclic ring, e.g., which is substituted or unsubstituted,

with the proviso that at least one of R_6, R_7, or R'_7 is present and includes an amine, e.g., as one of the atoms which makes up the ring;

R_8 represents an aryl, a cycloalkyl, a cycloalkenyl, a heterocycle, or a polycycle; and

m is an integer in the range 0 to 8 inclusive.

In certain embodiments, R_2 represents =O, sugar (e.g., monosaccharide, disaccharide, polysaccharide, etc.), carbamate (e.g., attached to the steroid at oxygen), ester (e.g., attached to the steroid at oxygen), carbonate, or alkoxy. Substituents such as carbamate, ester, carbonate, and alkoxy may be substituted or unsubstituted, e.g., may include additional functional groups such as aryl, aralkyl, heteroaryl, heteroaralkyl, amide, acylamino, carbonyl, ester, carbamate, urea, ketone, sulfonamide, etc.

In certain embodiments, the amine of R_6, R_7, or R'_7 is a tertiary amine.

In particular embodiments, R_3, for each occurrence, is an -OH, alkyl, -O-alkyl, -C(O)-alkyl, or -C(O)-R_8.

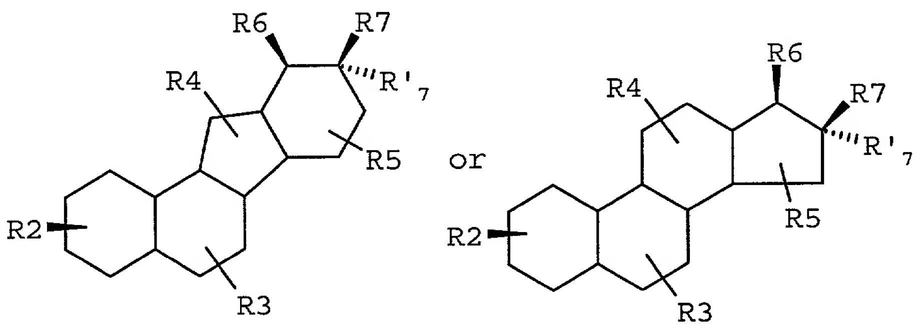
In particular embodiments, R_4, for each occurrence, is an absent, or represents -OH, =O, alkyl, -O-alkyl, -C(O)-alkyl, or -C(O)-R_8.

In particular embodiments, two of R_6, R_7, and R'_7 taken together form a nitrogen-containing ring, such as a furanopiperidine, such as perhydrofuro[3,2-b]pyridine, a pyranopiperidine, a quinoline, an indole, a pyranopyrrole, a naphthyridine, a thiofuranopiperidine, or a thiopyranopiperidine.

In certain embodiments, the nitrogen-containing ring comprises a tertiary amine, e.g., by having an extraannular substituent on the nitrogen atom, e.g., an alkyl substituted with, for example, aryl, aralkyl, heteroaryl, heteroaralkyl, amide, acylamino, carbonyl, ester, carbamate, urea, ketone, sulfonamide, etc. In certain embodiments, the extraannular substituent of the tertiary amine is a hydrophobic substituent. In certain embodiments, the hydrophobic extraannular substituent includes an aryl, heteroaryl, carbocyclyl, heterocyclyl, or polycyclyl group, such as biotin, a zwitterionic complex of boron, a steroidal polycycle, etc. In certain embodiments, the hydrophobic substituent may consist essentially of a combination of alkyl, amido, acylamino, ketone, ester, ether, halogen, alkenyl, alkynyl, aryl, aralkyl, urea, or similar functional groups, including between 5 and 40 non-hydrogen atoms, more preferably between 5 and 20 non-hydrogen atoms.

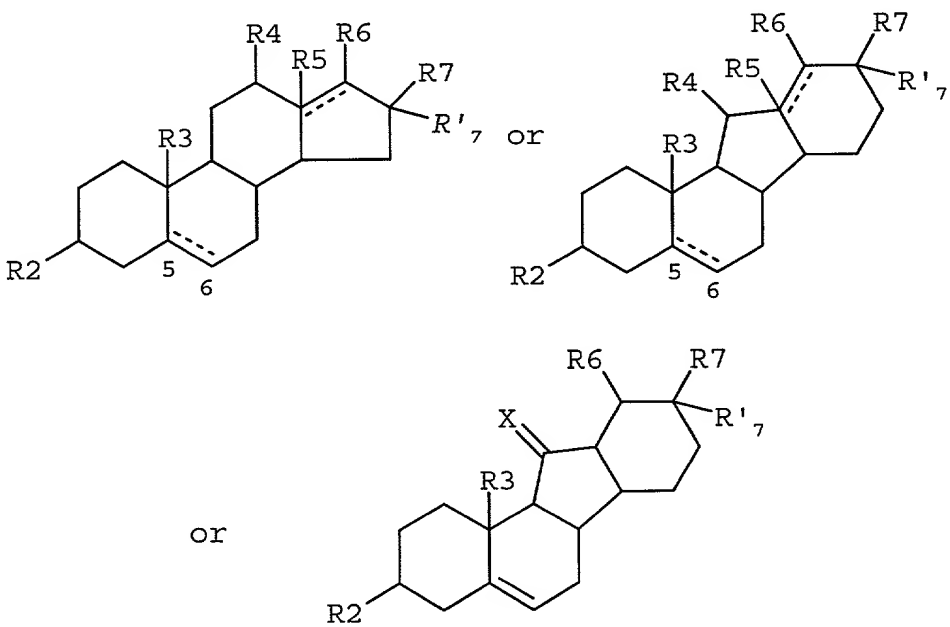
In particular embodiments, R_8 represents an aryl, a cycloalkyl, a cycloalkenyl, a heterocycle, or a polycycle, and preferably R_8 is a piperidine, pyrrolidine, pyridine, pyrimidine, morpholine, thiomorpholine, pyridazine, etc.

In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula Ia or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula Ia

In certain embodiments, the steroidal alkaloid is represented in the general formula (II), or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



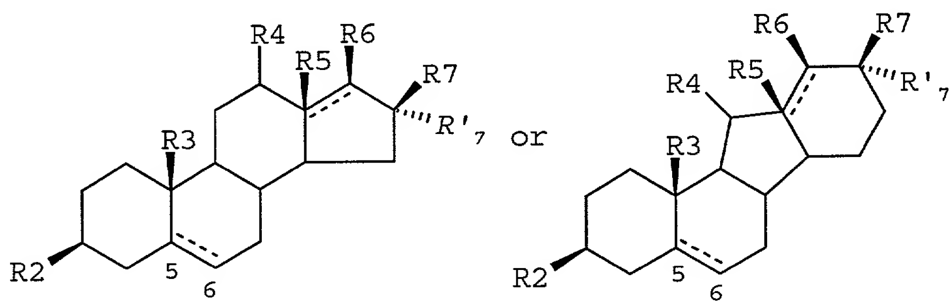
Formula II

wherein R_2, R_3, R_4, R_5, R_6, R_7, and R'_7 are as defined above, and X represents O or S, though preferably O.

In certain embodiments, R_2 represents =O, sugar (e.g., monosaccharide, disaccharide, polysaccharide, etc.), carbamate (e.g., attached to the steroid at oxygen), ester (e.g., attached to the steroid at oxygen), carbonate, or alkoxy. Substituents such as carbamate, ester, carbonate, and alkoxy may be substituted or unsubstituted, e.g., may include additional functional groups such as aryl, aralkyl, heteroaryl, heteroaralkyl, amide, acylamino, carbonyl, ester, carbamate, urea, ketone, sulfonamide, etc.

In certain embodiments, the amine of R_6, R_7, or R'_7 is a tertiary amine, e.g., substituted with a substituted or unsubstituted alkyl. In certain embodiments, the amine is part of a bicyclic ring system formed from R_7 and R'_7, e.g., a furanopiperidine system, and the third substituent is an alkyl substituted with, for example, aryl, aralkyl, heteroaryl, heteroaralkyl, amide, acylamino, carbonyl, ester, carbamate, urea, ketone, sulfonamide, etc. In certain embodiments, the extraannular substituent of the tertiary amine is a hydrophobic substituent. In certain embodiments, the hydrophobic extraannular substituent includes an aryl, heteroaryl, carbocyclyl, heterocyclyl, or polycyclyl group, such as biotin, a zwitterionic complex of boron, a steroidal polycycle, etc. In certain embodiments, the hydrophobic substituent may consist essentially of a combination of alkyl, amido, acylamino, ketone, ester, ether, halogen, alkenyl, alkynyl, aryl, aralkyl, urea, or similar functional groups, including between 5 and 40 non-hydrogen atoms, more preferably between 5 and 20 non-hydrogen atoms.

In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula IIa or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



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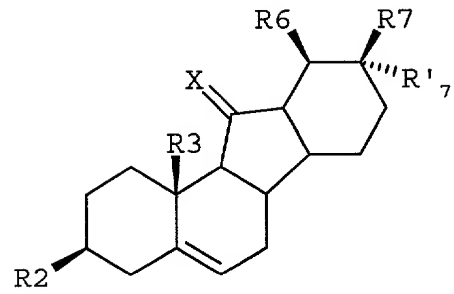
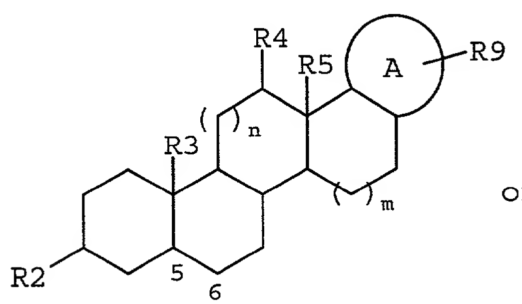


Formula IIa

In certain embodiments, the steroidal alkaloid is represented in the general formula (III), or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



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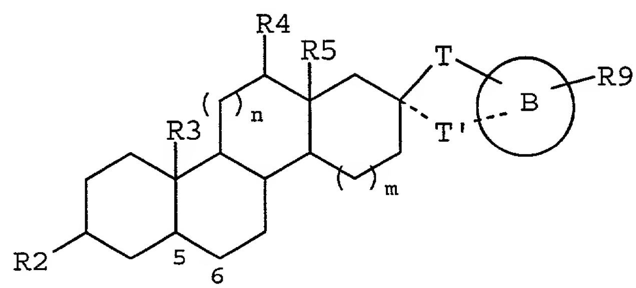


Formula III

wherein

R_2, R_3, R_4, R_5 and R_8 are as defined above;

A and B represent monocyclic or polycyclic groups;

T represents an alkyl, an aminoalkyl, a carboxyl, an ester, an amide, ether or amine linkage of 1-10 bond lengths;

T' is absent, or represents an alkyl, an aminoalkyl, a carboxyl, an ester, an amide, ether or amine linkage of 1-3 bond lengths, wherein if T and T' are present together, than T and T' taken together with the ring A or B form a covalently closed ring of 5-8 ring atoms;

R_9 represents one or more substitutions to the ring A or B, which for each occurrence, independently represent halogens, alkyls, alkenyls, alkynyls, aryls, hydroxyl, =O, =S, alkoxyl, silyloxy, amino, nitro, thiol, amines, imines, amides, phosphoryls, phosphonates, phosphines,

carbonyls, carboxyls, carboxamides, anhydrides, silyls, ethers, thioethers, alkylsulfonyls, arylsulfonyls, selenoethers, ketones, aldehydes, esters, or $-(CH_2)_m-R_8$; and

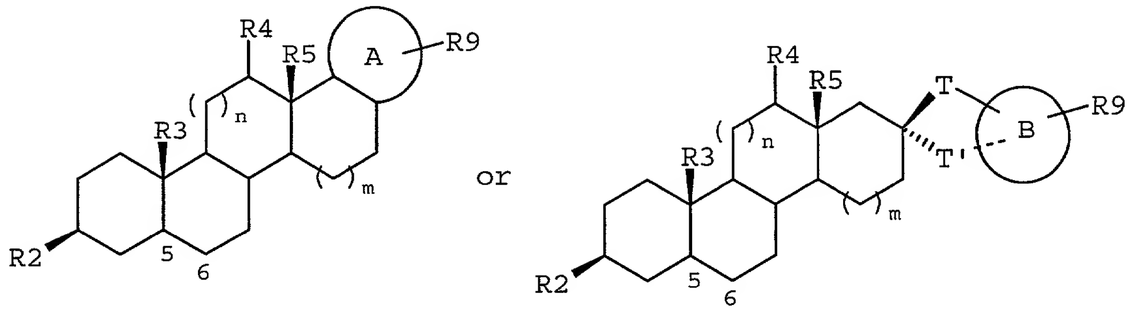
n and m are, independently, zero, 1 or 2;

with the proviso that A, or T, T', and B, taken together, include at least one amine.

In certain embodiments, R_2 represents =O, sugar (e.g., monosaccharide, disaccharide, polysaccharide, etc.), carbamate (e.g., attached to the steroid at oxygen), ester (e.g., attached to the steroid at oxygen), carbonate, or alkoxy. Substituents such as carbamate, ester, carbonate, and alkoxy may be substituted or unsubstituted, e.g., may include additional functional groups such as aryl, aralkyl, heteroaryl, heteroaralkyl, amide, acylamino, carbonyl, ester, carbamate, urea, ketone, sulfonamide, etc.

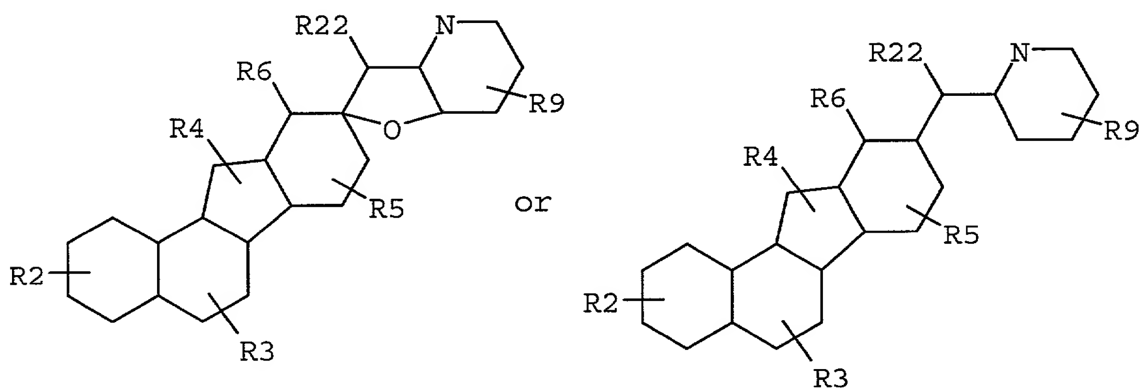
In certain embodiments, the amine of A, or T, T', and B, is a tertiary amine, e.g., substituted with a substituted or unsubstituted alkyl, e.g., substituted with aryl, aralkyl, heteroaryl, heteroaralkyl, amide, acylamino, carbonyl, ester, carbamate, urea, ketone, sulfonamide, etc. In certain embodiments, the extraannular substituent of the tertiary amine is a hydrophobic substituent. In certain embodiments, the hydrophobic extraannular substituent includes an aryl, heteroaryl, carbocyclyl, heterocyclyl, or polycyclyl group, such as biotin, a zwitterionic complex of boron, a steroidal polycycle, etc. In certain embodiments, the hydrophobic substituent may consist essentially of a combination of alkyl, amido, acylamino, ketone, ester, ether, halogen, alkenyl, alkynyl, aryl, aralkyl, urea, or similar functional groups, including between 5 and 40 non-hydrogen atoms, more preferably between 5 and 20 non-hydrogen atoms.

In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula IIIa or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula IIIa

For example, the subject methods can utilize *smoothened* antagonists based on the veratrum-type steroidal alkaloids jervine, cyclopamine, cycloposine, mukiamine or veratramine, e.g., which may be represented in the general formula (IV), or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula IV

wherein

R_2, R_3, R_4, R_5, R_6 and R_9 are as defined above;

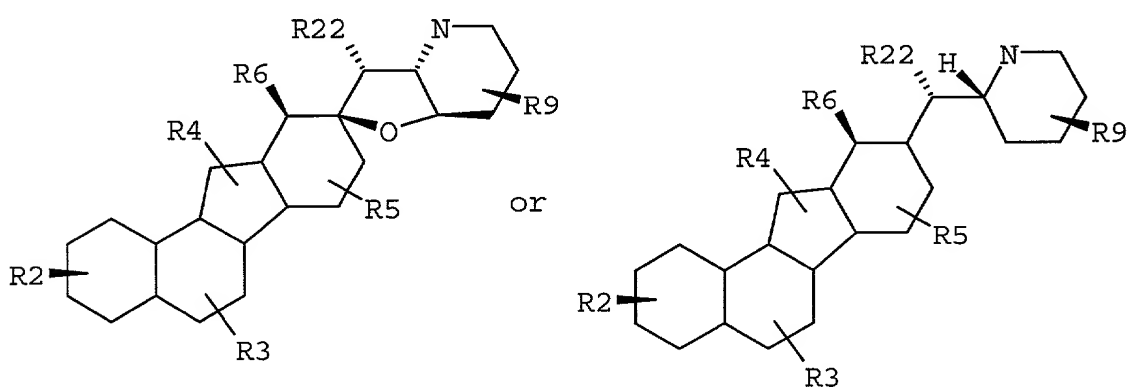
R_{22} is absent or represents an alkyl, an alkoxyl or -OH.

In certain embodiments, R_2 represents =O, sugar (e.g., monosaccharide, disaccharide, polysaccharide, etc.), carbamate (e.g., attached to the steroid at oxygen), ester (e.g., attached to the steroid at oxygen), carbonate, or alkoxy. Substituents such as carbamate, ester, carbonate, and alkoxy may be substituted or unsubstituted, e.g., may include additional functional groups

such as aryl, aralkyl, heteroaryl, heteroaralkyl, amide, acylamino, carbonyl, ester, carbamate, urea, ketone, sulfonamide, etc.

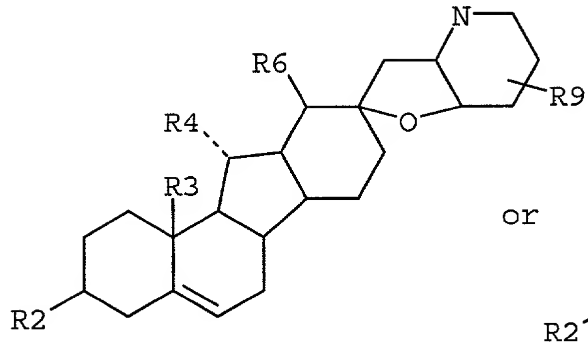
In certain embodiments, R_9 includes a substituent on nitrogen, e.g., a substituted or unsubstituted alkyl, e.g., substituted with, for example, aryl, aralkyl, heteroaryl, heteroaralkyl, amide, acylamino, carbonyl, ester, carbamate, urea, ketone, sulfonamide, etc. In certain embodiments, the extraannular substituent (e.g., R_9) of the tertiary amine is a hydrophobic substituent. In certain embodiments, the hydrophobic extraannular substituent includes an aryl, heteroaryl, carbocyclyl, heterocyclyl, or polycyclyl group, such as biotin, a zwitterionic complex of boron, a steroidal polycycle, etc. In certain embodiments, the hydrophobic substituent may consist essentially of a combination of alkyl, amido, acylamino, ketone, ester, ether, halogen, alkenyl, alkynyl, aryl, aralkyl, urea, or similar functional groups, including between 5 and 40 non-hydrogen atoms, more preferably between 5 and 20 non-hydrogen atoms.

In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula IVa or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula IVa

In certain embodiments, the steroidal alkaloid is represented in the general formula (V) or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



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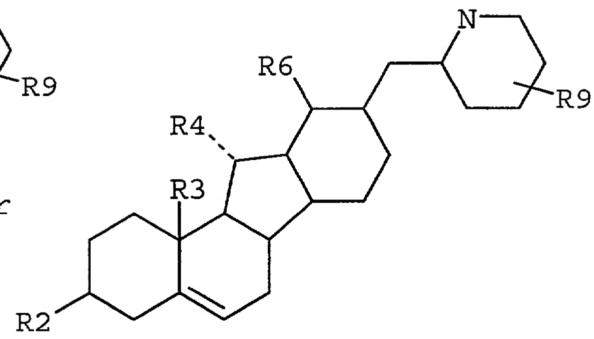

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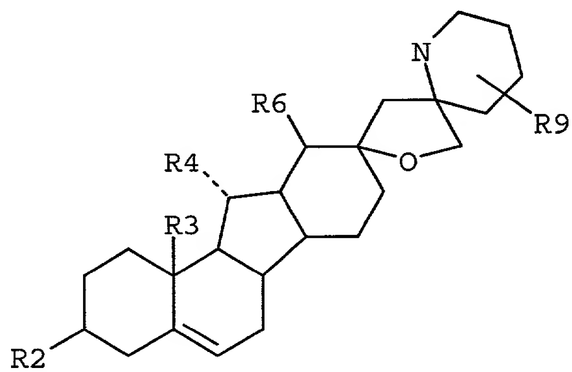


Formula V

wherein R_2, R_3, R_4, R_6 and R_9 are as defined above;

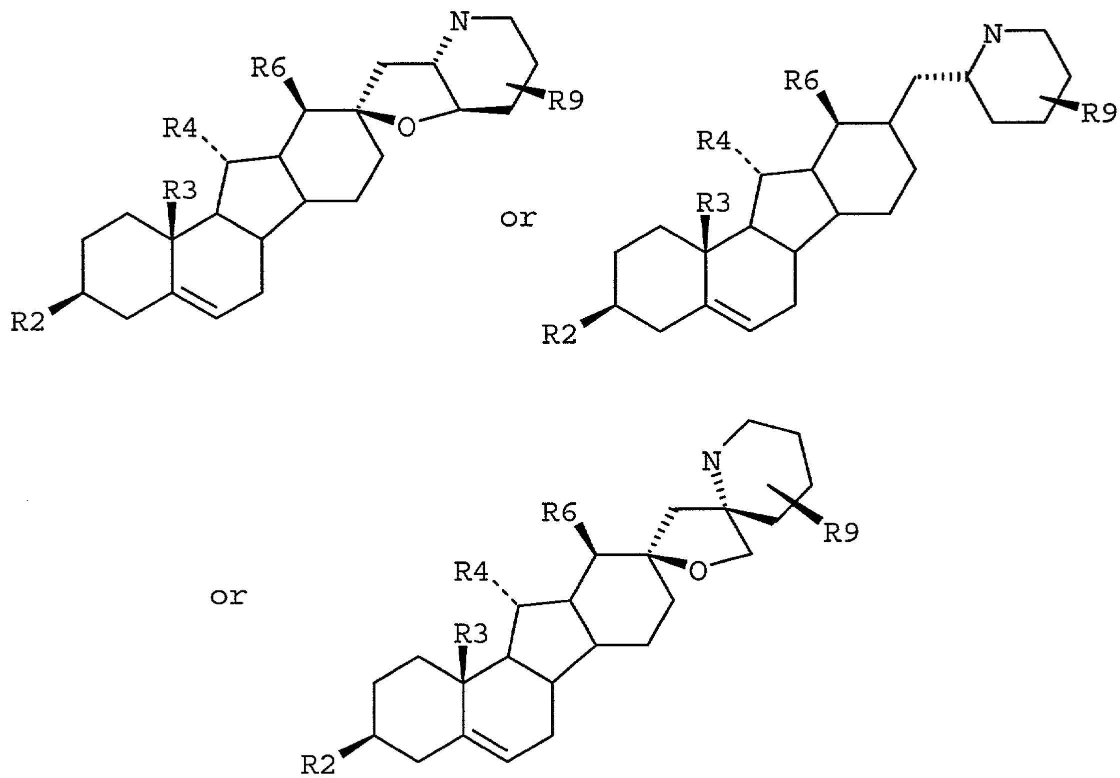
In certain embodiments, R_2 represents =O, sugar (e.g., monosaccharide, disaccharide, polysaccharide, etc.), carbamate (e.g., attached to the steroid at oxygen), ester (e.g., attached to the steroid at oxygen), carbonate, or alkoxy. Substituents such as carbamate, ester, carbonate, and alkoxy may be substituted or unsubstituted, e.g., may include additional functional groups such as aryl, aralkyl, heteroaryl, heteroaralkyl, amide, acylamino, carbonyl, ester, carbamate, urea, ketone, sulfonamide, etc.

In certain embodiments, R_9 includes a substituent on nitrogen, e.g., a substituted or unsubstituted alkyl, e.g., substituted with, for example, aryl, aralkyl, heteroaryl, heteroaralkyl, amide, acylamino, carbonyl, ester, carbamate, urea, ketone, sulfonamide, etc.

In certain embodiments, the extraannular substituent of the tertiary amine (e.g., R_9) is a hydrophobic substituent. In certain embodiments, the hydrophobic extraannular substituent includes an aryl, heteroaryl, carbocyclyl, heterocyclyl, or polycyclyl group, such as biotin, a zwitterionic complex of boron, a steroidal polycycle, etc. In certain embodiments, the

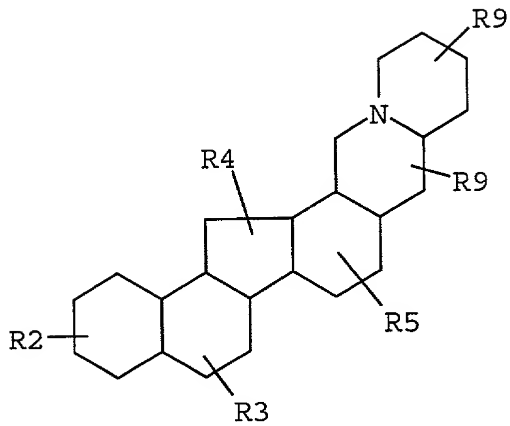
hydrophobic substituent may consist essentially of a combination of alkyl, amido, acylamino, ketone, ester, ether, halogen, alkenyl, alkynyl, aryl, aralkyl, urea, or similar functional groups, including between 5 and 40 non-hydrogen atoms, more preferably between 5 and 20 non-hydrogen atoms.

In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula Va or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula Va

Another class of *smoothened* antagonists can be based on the veratrum-type steroidal alkaloids resembling verticine and zygacine, e.g., general formula (VI), or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



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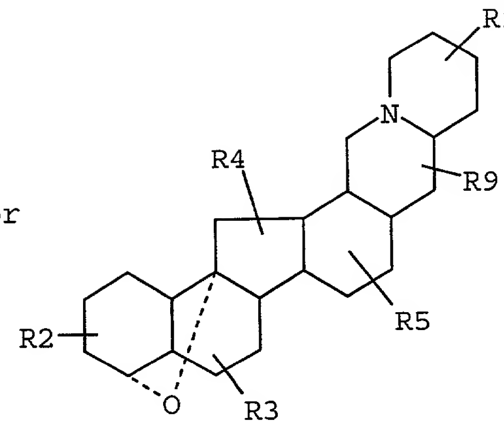
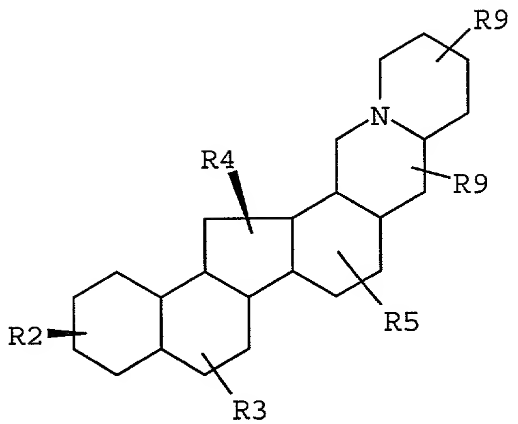


Formula VI

wherein R_2, R_3, R_4, R_5 and R_9 are as defined above;

In certain embodiments, R_2 represents =O, sugar (e.g., monosaccharide, disaccharide, polysaccharide, etc.), carbamate (e.g., attached to the steroid at oxygen), ester (e.g., attached to the steroid at oxygen), carbonate, or alkoxy. Substituents such as carbamate, ester, carbonate, and alkoxy may be substituted or unsubstituted, e.g., may include additional functional groups such as aryl, aralkyl, heteroaryl, heteroaralkyl, amide, acylamino, carbonyl, ester, carbamate, urea, ketone, sulfonamide, etc.

In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula VIa or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



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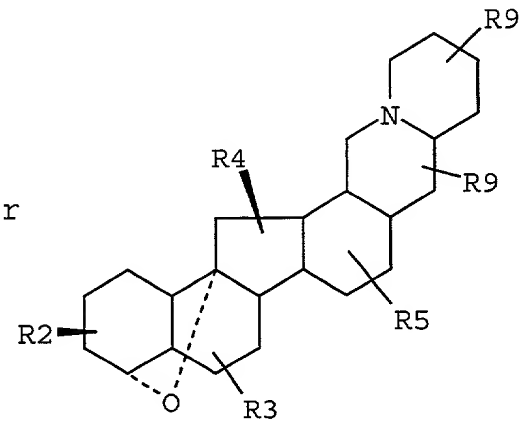
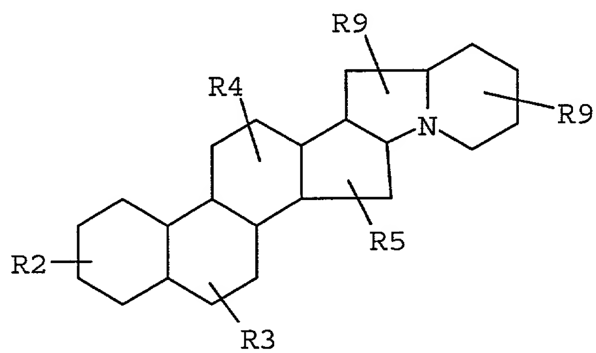


Formula VIa

In certain embodiments, the steroidal alkaloid is represented in the general formula (VII) or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:

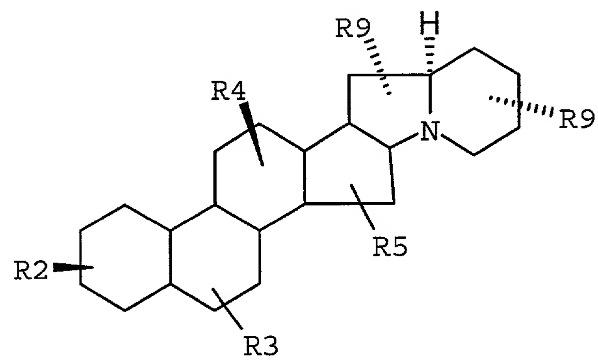


Formula VII

wherein R_2, R_3, R_4, R_5 and R_9 are as defined above.

In certain embodiments, R_2 represents =O, sugar (e.g., monosaccharide, disaccharide, polysaccharide, etc.), carbamate (e.g., attached to the steroid at oxygen), ester (e.g., attached to the steroid at oxygen), carbonate, or alkoxy. Substituents such as carbamate, ester, carbonate, and alkoxy may be substituted or unsubstituted, e.g., may include additional functional groups such as aryl, aralkyl, heteroaryl, heteroaralkyl, amide, acylamino, carbonyl, ester, carbamate, urea, ketone, sulfonamide, etc.

In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula VIIa or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula VIIa

In certain embodiments, the subject antagonists and activators can be chosen on the basis of their selectively for the *smoothened* pathway. This selectivity can be for the *smoothened* pathway versus other steroid-mediated pathways (such as testosterone or estrogen mediated activities), as well as selectivity for particular *hedgehog/ptc/smoothened* pathways, e.g., which isotype specific for *ptc* (e.g., *ptc*-1, *ptc*-2) or *hedgehog* (e.g., *Shh*, *Ihh*, *Dhh*, etc.). For instance, the subject method may employ steroidal alkaloids which do not substantially interfere with the biological activity of such steroids as aldosterone, androstane, androstene, androstenedione, androsterone, cholecalciferol, cholestane, cholic acid, corticosterone, cortisol, cortisol acetate, cortisone, cortisone acetate, deoxycorticosterone, digitoxigenin, ergocalciferol, ergosterol, estradiol-17-α, estradiol-17-β, estriol, estrane, estrone, hydrocortisone, lanosterol, lithocholic acid, mestranol, β-methasone, prednisone, pregnane, pregnenolone, progesterone, spironolactone, testosterone, triamcinolone and their derivatives, at least so far as those activities are unrelated to *ptc* related signaling.

In one embodiment, the subject steroidal alkaloid for use in the present method has a k_d for members of the nuclear hormone receptor superfamily of greater than 1 μM, and more preferably greater than 1 mM, e.g., it does not bind estrogen, testosterone receptors or the like.

Preferably, the subject *smoothened* antagonist has no estrogenic activity at physiological concentrations (e.g., in the range of 1 ng -1 mg/kg).

In this manner, untoward side effects which may be associated certain members of the steroidal alkaloid class can be reduced. For example, using the drug screening assays described herein, the application of combinatorial and medicinal chemistry techniques to the steroidal alkaloids provides a means for reducing such unwanted negative side effects including personality changes, shortened life spans, cardiovascular diseases and vascular occlusion, organ toxicity, hyperglycemia and diabetes, Cushnoid features, "wasting" syndrome, steroidal glaucoma, hypertension, peptic ulcers, and increased susceptibility to infections. For certain embodiments, it will be beneficial to reduce the teratogenic activity relative to jervine, as for example, in the use of the subject method to selectively inhibit spermatogenesis.

In a preferred embodiment, the subject antagonists are steroidal alkaloids other than spirosolane, tomatidine, jervine, etc.

In particular embodiments, the steroidal alkaloid is chosen for use because it is more selective for one *patched* isoform over the next, e.g., 10-fold, and more preferably at least 100- or even 1000-fold more selective for one *patched* pathway (*ptc*-1, *ptc*-2) over another. Likewise, the steroidal alkaloid may be chosen for use because it is more selective for one *smoothened* isoform over the next, e.g., 10-fold, and more preferably at least 100- or even 1000-fold more selective for one wild-type *smoothened* protein (should various isoforms exist) or for activated *smoothened* mutants relative to wild-type *smoothened*. In certain embodiments, the subject method can be carried out conjointly with the administration of growth and/or trophic factors, or compositions that also act on other parts of the *hedgehog/smoothened* pathway.. For instance, it is contemplated that the subject methods can include treatment with an agent that modulates cAMP levels, e.g., increasing or decreasing intracellular levels of cAMP.

In one embodiment, the subject method utilizes a *smoothened* antagonist, and the conjoint agent elevates cAMP levels in order to enhance the efficacy of the *smoothened* antagonist.

For example, compounds that may activate adenylate cyclase include forskolin (FK), cholera toxin (CT), pertussis toxin (PT), prostaglandins (e.g., PGE-1 and PGE-2), colforsin and β-adrenergic receptor agonists. β-Adrenergic receptor agonists (sometimes referred to herein as "β-adrenergic agonists") include albuterol, bambuterol, bitolterol, carbuterol, clenbuterol,

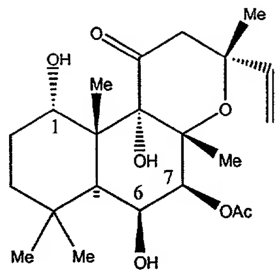
clorprenaline, denopamine, dioxethedrine, dopexamine, ephedrine, epinephrine, etafedrine, ethylnorepinephrine, fenoterol, formoterol, hexoprenaline, ibopamine, isoetharine, isoproterenol, mabuterol, metaproterenol, methoxyphenamine, norepinephrine, oxyfedrine, pirbuterol, prenalterol, procaterol, propranolol, protokylol, quinterenol, reproterol, rimiterol, ritodrine, salmefamol, soterenol, salmeterol, terbutaline, tretoquinol, tulobuterol, and xamoterol.

Compounds which may inhibit a cAMP phosphodiesterase include amrinone, milrinone, xanthine, methylxanthine, anagrelide, cilostamide, medorinone, indolidan, rolipram, 3-isobutyl-1-methylxanthine (IBMX), chelerythrine, cilostazol, glucocorticoids, griseolic acid, etazolate, caffeine, indomethacin, papverine, MDL 12330A, SQ 22536, GDPssS, clonidine, type III and type IV phosphodiesterase inhibitors, methylxanthines such as pentoxifylline, theophylline, theobromine, pyrrolidinones and phenyl cycloalkane and cycloalkene derivatives (described in PCT publications Nos. WO 92/19594 and WO 92/10190), lisophylline, and fenoxamine.

Analogs of cAMP which may be useful in the present method include dibutyryl-cAMP (db-cAMP), (8-(4)-chlorophenylthio)-cAMP (cpt-cAMP), 8-[(4-bromo-2,3-dioxobutyl)thio]-cAMP, 2-[(4-bromo-2,3-dioxobutyl)thio]-cAMP, 8-bromo-cAMP, dioctanoyl-cAMP, Sp-adenosine 3':5'-cyclic phosphorothioate, 8-piperidino-cAMP, N^6-phenyl-cAMP, 8-methylamino-cAMP, 8-(6-aminohexyl)amino-cAMP, 2'-deoxy-cAMP, N^6,2'-O-dibutryl-cAMP, N^6,2'-O-disuccinyl-cAMP, N^6-monobutyryl-cAMP, 2'-O-monobutyryl-cAMP, 2'-O-monobutryl-8-bromo-cAMP, N^6-monobutryl-2'-deoxy-cAMP, and 2'-O-monosuccinyl-cAMP.

Compounds which may reduce the levels or activity of cAMP include prostaglandylinositol cyclic phosphate (cyclic PIP), endothelins (ET)-1 and -3, norepinepurine, K252a, dideoxyadenosine, dynorphins, melatonin, pertussis toxin, staurosporine, G_i agonists, MDL 12330A, SQ 22536, GDPssS and clonidine, beta-blockers, and ligands of G-protein coupled receptors. Additional compounds are disclosed in U.S. Patent Nos. 5,891,875, 5,260,210, and 5,795,756.

Above-listed compounds useful in the subject methods may be modified to increase the bioavailability, activity, or other pharmacologically relevant property of the compound. For example, forskolin has the formula:



Forskolin

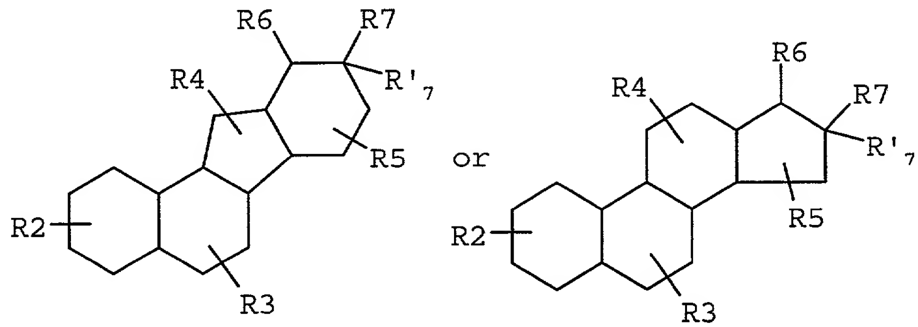
Modifications of forskolin that have been found to increase the hydrophilic character of forskolin without severely attenuating the desired biological activity include acylation of the hydroxyls at C6 and/or C7 (after removal of the acetyl group) with hydrophilic acyl groups. In compounds wherein C6 is acylated with a hydrophilic acyl group, C7 may optionally be deacetylated. Suitable hydrophilic acyl groups include groups having the structure $-(CO)(CH_2)_nX$, wherein X is OH or NR_2; R is hydrogen, a C_1-C_4 alkyl group, or two Rs taken together form a ring comprising 3-8 atoms, preferably 5-7 atoms, which may include heteroatoms (e.g., piperazine or morpholine rings); and n is an integer from 1-6, preferably from 1-4, even more preferably from 1-2. Other suitable hydrophilic acyl groups include hydrophilic amino acids or derivatives thereof, such as aspartic acid, glutamic acid, asparagine, glutamine, serine, threonine, tyrosine, etc., including amino acids having a heterocyclic side chain. Forskolin, or other compounds listed above, modified by other possible hydrophilic acyl side chains known to those of skill in the art may be readily synthesized and tested for activity in the present method.

Similarly, variants or derivatives of any of the above-listed compounds may be effective as cAMP antagonists in the subject method, e.g., in order to decrease cAMP levels and potentiate the activity of a *smoothened* activator. Those skilled in the art will readily be able to synthesize and test such derivatives for suitable activity.

Exemplary compounds part II:

Additional steroidal alkaloids are contemplated as potential *hedgehog antag*onists for use in the subject method. For example, compounds useful in the subject methods include steroidal

alkaloids represented in the general formulas (VIII), or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula VIII

wherein, as valence and stability permit,

R_2, R_3, R_4, and R_5, represent one or more substitutions to the ring to which each is attached, for each occurrence, independently represent hydrogen, halogens, alkyls, alkenyls, alkynyls, aryls, hydroxyl, =O, =S, alkoxyl, silyloxy, amino, nitro, thiol, amines, imines, amides, phosphoryls, phosphonates, phosphines, carbonyls, carboxyls, carboxamides, anhydrides, silyls, ethers, thioethers, alkylsulfonyls, arylsulfonyls, selenoethers, ketones, aldehydes, esters, sugar (e.g., monosaccharide, disaccharide, polysaccharide, etc.), carbamate (e.g., attached to the steroid at oxygen), carbonate, or $-(CH_2)_m-R_8$;

R_6, R_7, and R'_7, are absent or represent, independently, halogens, alkyls, alkenyls, alkynyls, aryls, hydroxyl, =O, =S, alkoxyl, silyloxy, amino, nitro, thiol, amines, imines, amides, phosphoryls, phosphonates, phosphines, carbonyls, carboxyls, carboxamides, anhydrides, silyls, ethers, thioethers, alkylsulfonyls, arylsulfonyls, selenoethers, ketones, aldehydes, esters, or $-(CH_2)_m-R_8$, or

R_6 and R_7, or R_7 and R'_7, taken together form a ring or polycyclic ring, e.g., which is substituted or unsubstituted,

with the proviso that at least one of R_6, R_7, or R'_7 is present and includes a primary or secondary amine;

R_8 represents an aryl, a cycloalkyl, a cycloalkenyl, a heterocycle, or a polycycle; and

m is an integer in the range 0 to 8 inclusive.

In preferred embodiments,

R_2 and R_3, for each occurrence, is an -OH, alkyl, -O-alkyl, -C(O)-alkyl, or -C(O)-R_8;

R_4, for each occurrence, is an absent, or represents -OH, =O, alkyl, -O-alkyl, -C(O)-alkyl, or -C(O)-R_8;

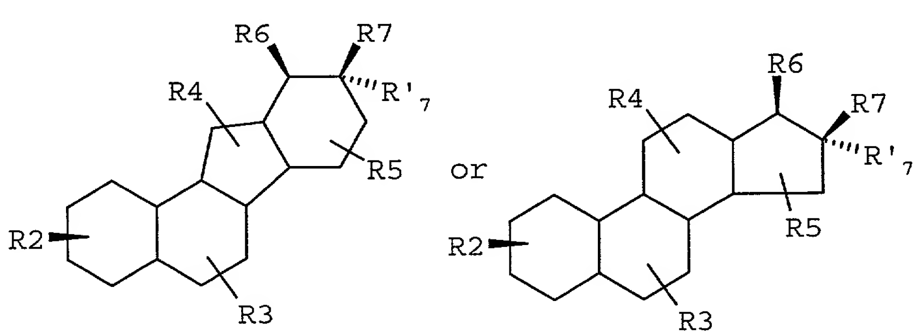
R_6, R_7, and R'_7 each independently represent, hydrogen, alkyls, alkenyls, alkynyls, amines, imines, amides, carbonyls, carboxyls, carboxamides, ethers, thioethers, esters, or -$(CH_2)_m$-R_8, or

R_7, and R'_7 taken together form a furanopiperidine, such as perhydrofuro[3,2-b]pyridine, a pyranopiperidine, a quinoline, an indole, a pyranopyrrole, a naphthyridine, a thiofuranopiperidine, or a thiopyranopiperidine

with the proviso that at least one of R_6, R_7, or R'_7 is present and includes a primary or secondary amine;

R_8 represents an aryl, a cycloalkyl, a cycloalkenyl, a heterocycle, or a polycycle, and preferably R_8 is a piperidine, pyrimidine, morpholine, thiomorpholine, pyridazine,

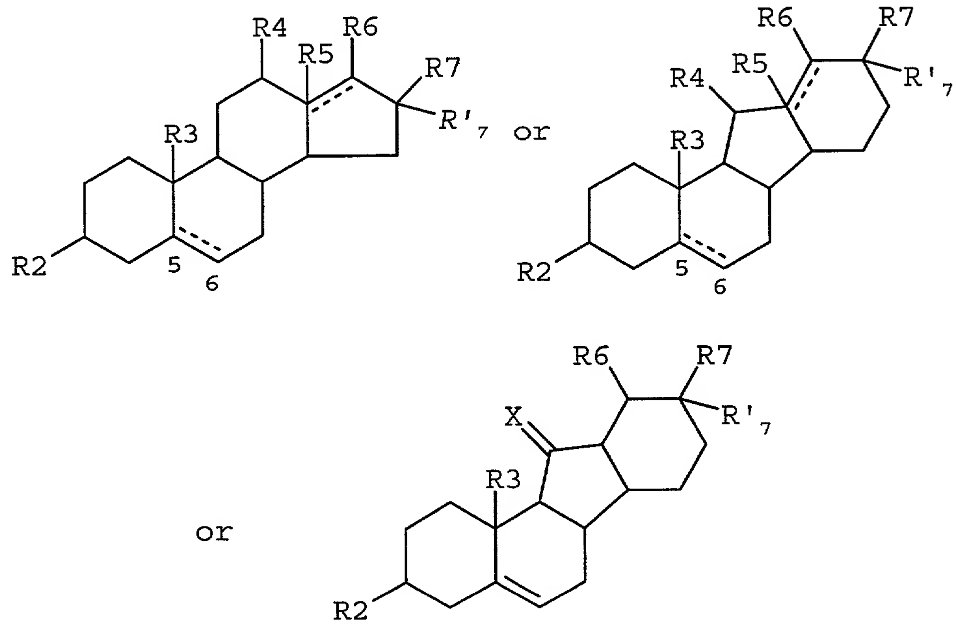
In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula VIIIa or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula VIIIa

In preferred embodiments, the subject *hedgehog* antagonists can be represented in one of the following general formulas (IX) or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:

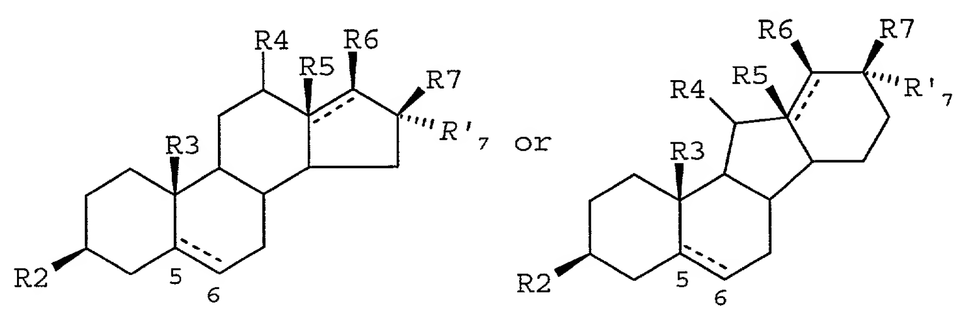
:



Formula IX

wherein R_2, R_3, R_4, R_5, R_6, R_7, and R'_7 are as defined above, and X represents O or S, though preferably O.

In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula IXa or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



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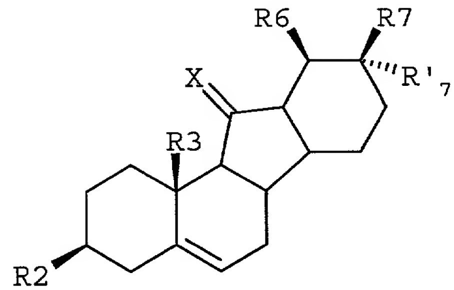
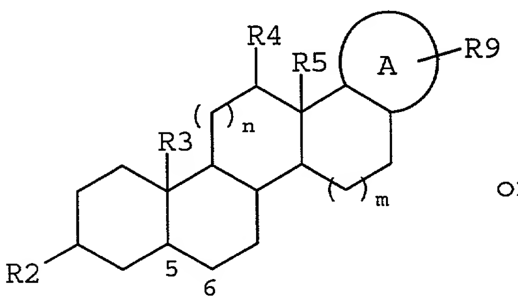


Formula IXa

In certain embodiments, the subject *hedgehog* antagonists are represented by the general formula (X) or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



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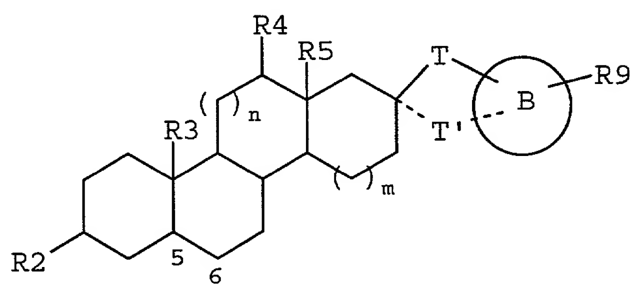


Formula X

wherein

R_2, R_3, R_4, R_5 and R_8 are as defined above;

A and B represent monocyclic or polycyclic groups;

T represents an alkyl, an aminoalkyl, a carboxyl, an ester, an amide, ether or amine linkage of 1-10 bond lengths;

T' is absent, or represents an alkyl, an aminoalkyl, a carboxyl, an ester, an amide, ether or amine linkage of 1-3 bond lengths, wherein if T and T' are present together, than T and T' taken together with the ring A or B form a covalently closed ring of 5-8 ring atoms;

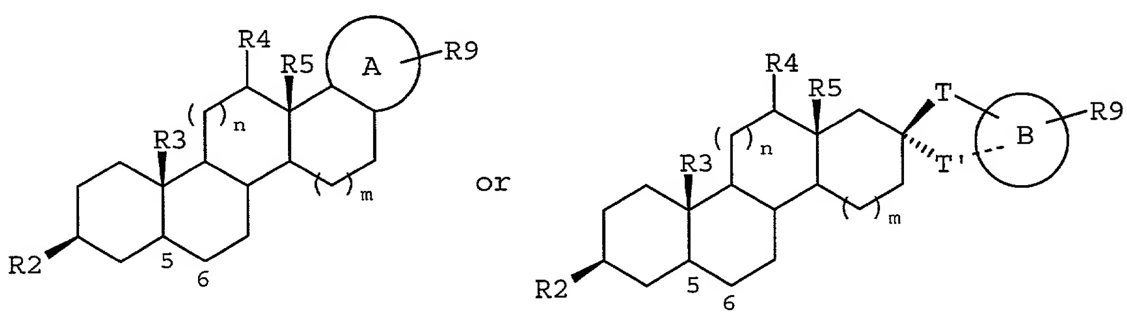
R9 represents one or more substitutions to the ring A or B, which for each occurrence, independently represent halogens, alkyls, alkenyls, alkynyls, aryls, hydroxyl, =O, =S, alkoxyl, silyloxy, amino, nitro, thiol, amines, imines, amides, phosphoryls, phosphonates, phosphines,

carbonyls, carboxyls, carboxamides, anhydrides, silyls, ethers, thioethers, alkylsulfonyls, arylsulfonyls, selenoethers, ketones, aldehydes, esters, or $-(CH_2)_m-R_8$; and

n and m are, independently, zero, 1 or 2;

with the proviso that A and R_9, or T, T' B and R_9, taken together include at least one primary or secondary amine.

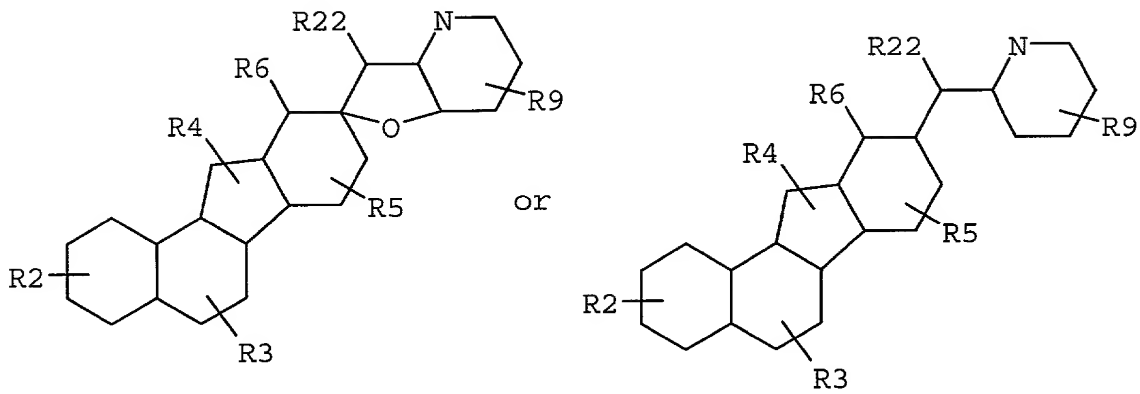
In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula Xa or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula Xa

For example, the subject methods can utilize *hedgehog* antagonists based on the veratrum-type steroidal alkaloids jervine, cyclopamine, cycloposine, mukiamine or veratramine, e.g., which may be represented in the general formula (XI) or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:

:



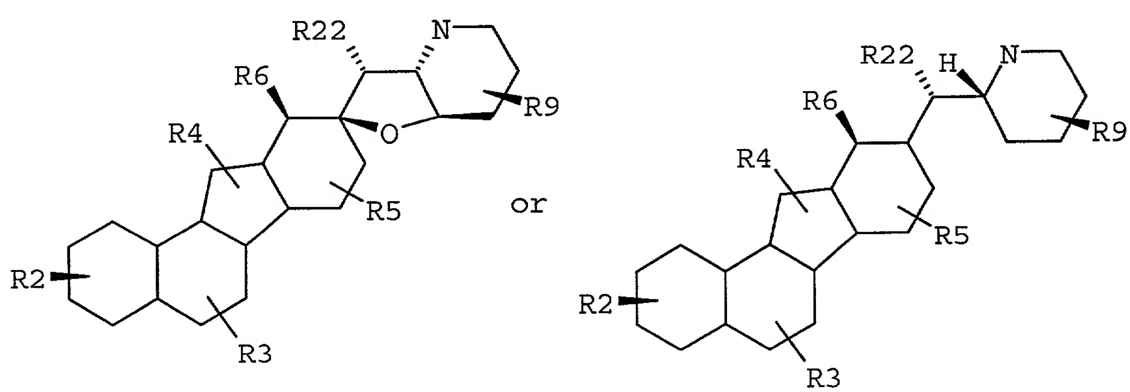
Formula XI

wherein

R_2, R_3, R_4, R_5, R_6 and R_9 are as defined above;

R_{22} is absent or represents an alkyl, an alkoxyl or -OH.

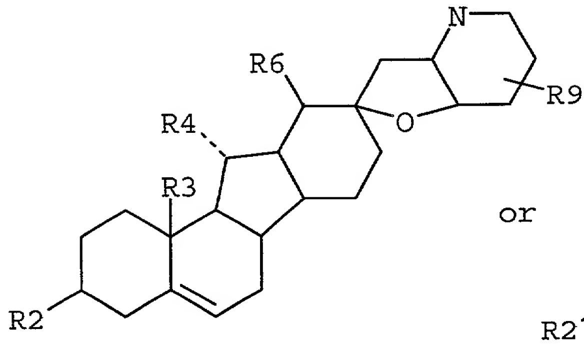
In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula XIa or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



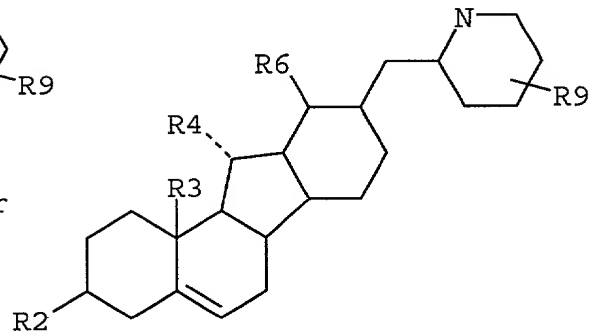
Formula XIa

In even more preferred embodiments, the subject antagonists are represented in the formulas (XII) or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:

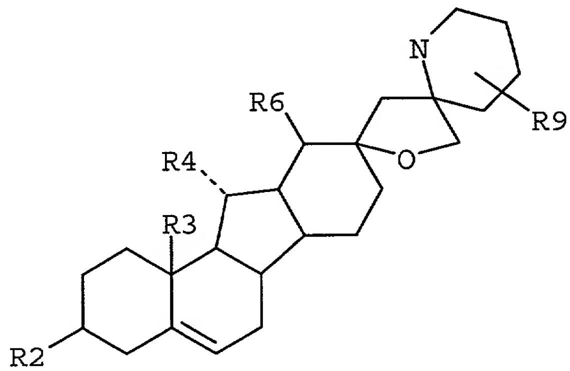
:



or



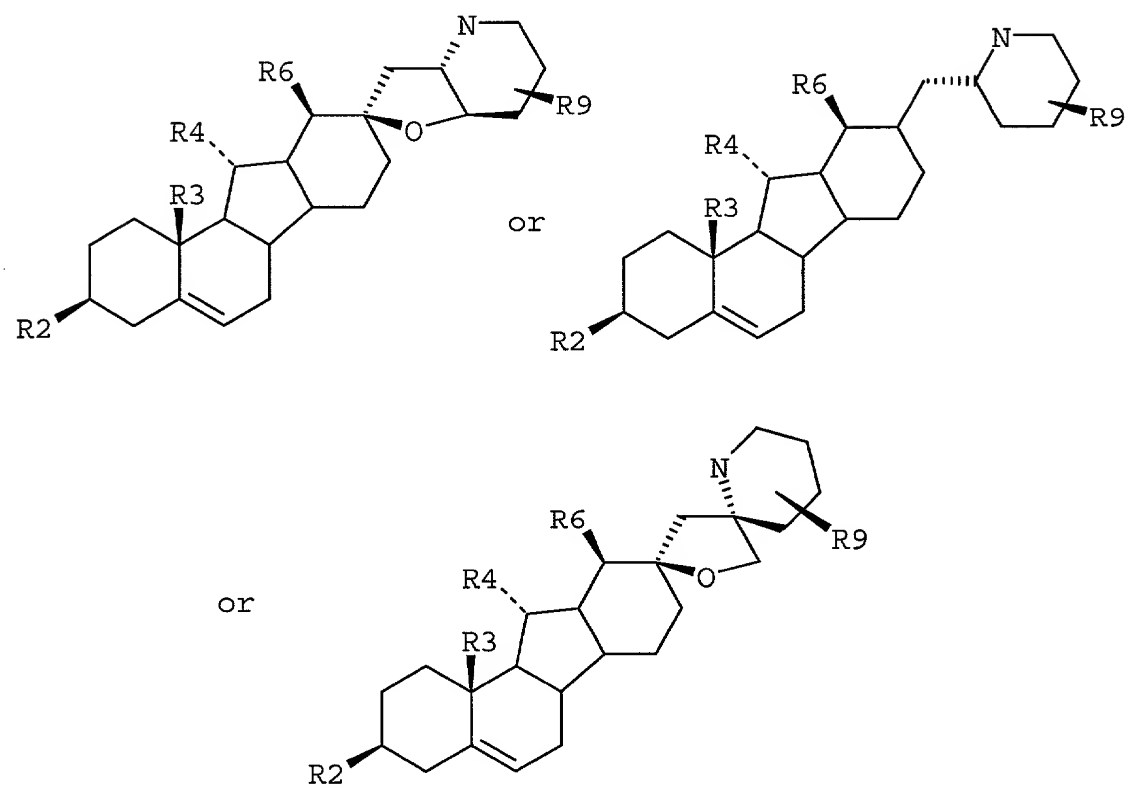
or



Formula XII

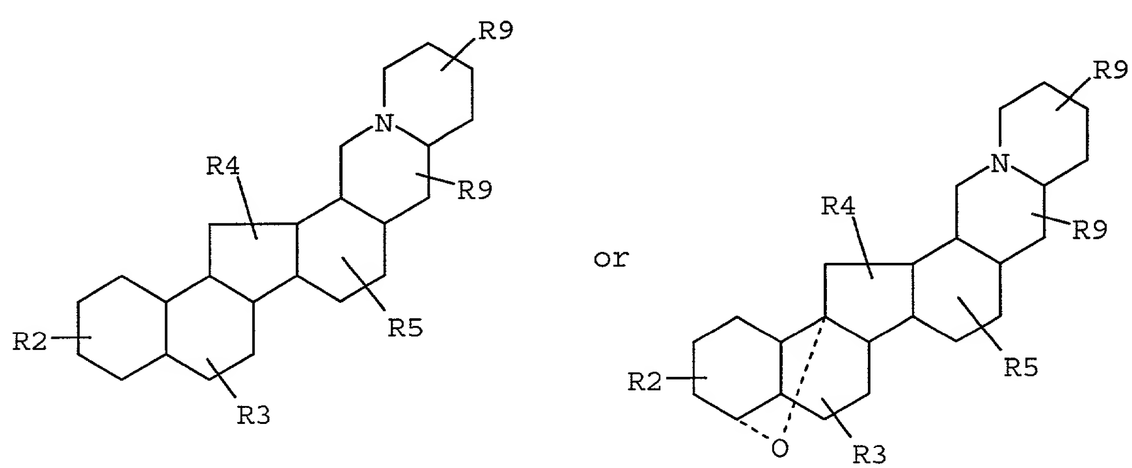
wherein R_2, R_3, R_4, R_6 and R_9 are as defined above;

In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula XIIa or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula XIIa

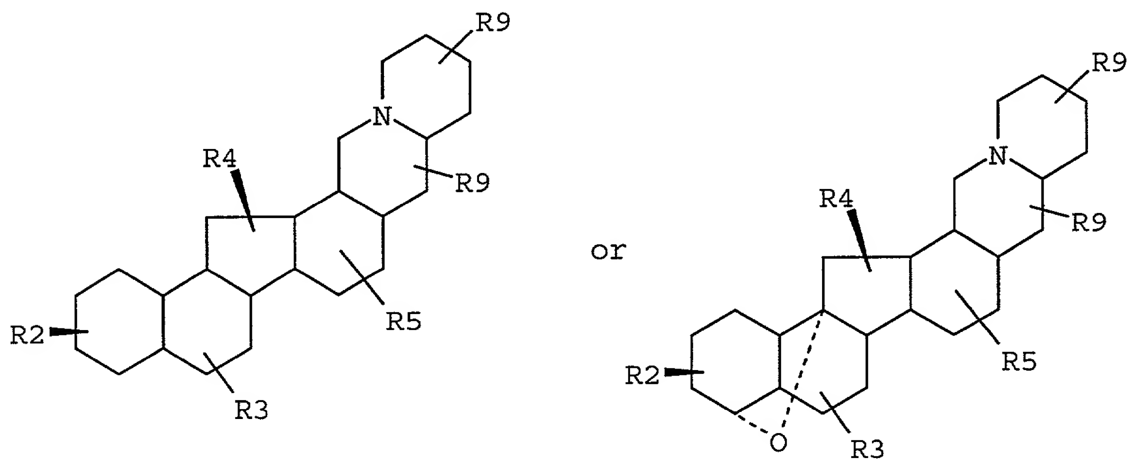
Another class of *hedgehog* antagonists can be based on the veratrum-type steroidal alkaloids resembling verticine and zygacine, e.g., represented in the general formulas (VI) or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula XIII

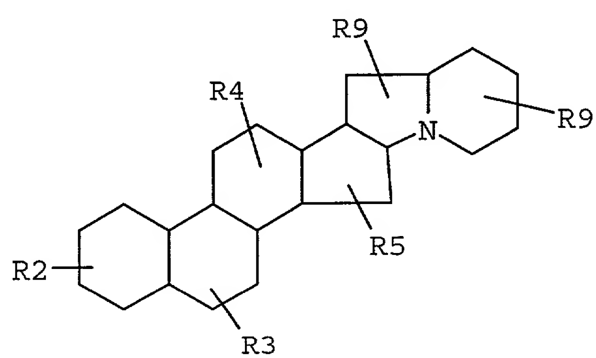
wherein R_2, R_3, R_4, R_5 and R_9 are as defined above.

In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula XIIIa or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula XIIIa

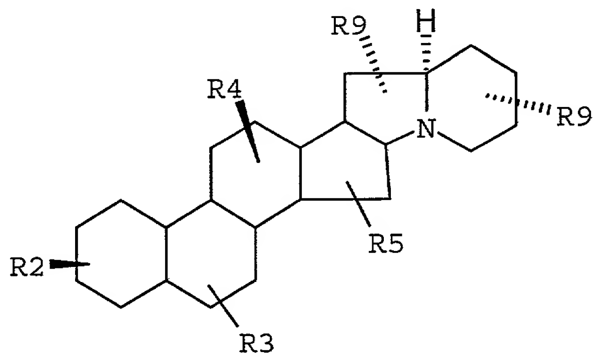
Still another class of potential *hedgehog* antagonists are based on the solanum-type steroidal alkaloids, e.g., solanidine, which may be represented in the general formula (XIV) or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula XIV

wherein R_2, R_3, R_4, R_5 and R_9 are as defined above.

In certain preferred embodiments, the definitions outlined above apply, and the subject compounds are represented by general formula XIVa or unsaturated forms thereof and/or seco-, nor- or homo-derivatives thereof:



Formula XIVa

In certain embodiments, the subject antagonists can be chosen on the basis of their selectively for the *hedgehog* pathway. This selectivity can for the *hedgehog* pathway versus other steroid-mediated pathways (such as testosterone or estrogen mediated activities), as well as selectivity for particular *hedgehog* pathways, e.g., which isotype specific for *hedgehog* (e.g., Shh, Ihh, Dhh) or the *patched* receptor (e.g., *ptc*-1, *ptc*-2). For instance, the subject method may employ steroidal alkaloids which do not substantially interfere with the biological activity of such steroids as aldosterone, androstane, androstene, androstenedione, androsterone, cholecalciferol, cholestane, cholic acid, corticosterone, cortisol, cortisol acetate, cortisone, cortisone acetate, deoxycorticosterone, digitoxigenin, ergocalciferol, ergosterol, estradiol-17-α, estradiol-17-β, estriol, estrane, estrone, hydrocortisone, lanosterol, lithocholic acid, mestranol, β-methasone, prednisone, pregnane, pregnenolone, progesterone, spironolactone, testosterone, triamcinolone and their derivatives, at least so far as those activities are unrelated to *ptc* related signaling.

In one embodiment, the subject steroidal alkaloid for use in the present method has a k_d for members of the nuclear hormone receptor superfamily of greater than 1 μM, and more preferably greater than 1 mM, e.g., it does not bind estrogen, testosterone receptors or the like.

Preferably, the subject *hedgehog antagonist* has no estrogenic activity at physiological concentrations (e.g., in the range of 1 ng -1 mg/kg).

In this manner, untoward side effects which may be associated certain members of the steroidal alkaloid class can be reduced. For example, using the drug screening assays described herein, the application of combinatorial and medicinal chemistry techniques to the steroidal alkaloids provides a means for reducing such unwanted negative side effects including personality changes, shortened life spans, cardiovascular diseases and vascular occlusion, organ toxicity, hyperglycemia and diabetes, Cushnoid features, "wasting" syndrome, steroidal glaucoma, hypertension, peptic ulcers, and increased susceptibility to infections. For certain embodiments, it will be beneficial to reduce the teratogenic activity relative to jervine, as for example, in the use of the subject method to selectively inhibit spermatogenesis.

In a preferred embodiment, the subject antagonists are steroidal alkaloids other than spirosolane, tomatidine, jervine, etc.

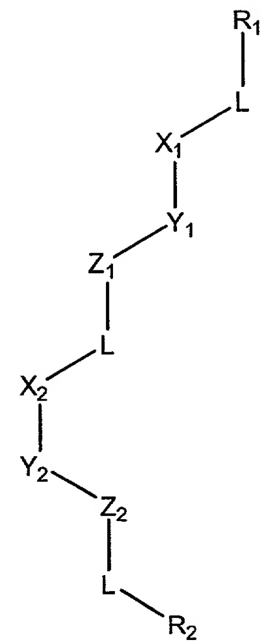
In certain preferred embodiments, the subject inhibitors inhibit a *hedgehog* signal transduction pathway with an ED_{50} of 1mM or less, more preferably of 1 μM or less, and even more preferably of 1nM or less.

In certain embodiments, the subject inhibitors inhibit a *hedgehog* signal transduction pathway with an ED_{50} of 1mM or less, more preferably 1 μM or less, and even more preferably 1nM or less.

In particular embodiments, the steroidal alkaloid is chosen for use because it is more selective for one *patched* isoform over the next, e.g., 10-fold, and more preferably at least 100- or even 1000-fold more selective for one *patched* pathway (*ptc*-1, *ptc*-2) over another.

Exemplary compounds part III:

As described in further detail below, it is contemplated that the subject methods can be carried out using a variety of different small molecules which can be readily identified, for example, by such drug screening assays as described herein. For example, compounds useful in the subject methods include compounds may be represented by general formula (XV):



Formula XV

wherein, as valence and stability permit,

R_1 and R_2, independently for each occurrence, represent H, lower alkyl, aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., $-(CH_2)_n$aryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., $-(CH_2)_n$heteroaralkyl-);

L, independently for each occurrence, is absent or represents $-(CH_2)_n$-alkyl, -alkenyl-, -alkynyl-, $-(CH_2)_n$alkenyl-, $-(CH_2)_n$alkynyl-, $-(CH_2)_nO(CH_2)_p-$, $-(CH_2)_nNR_2(CH_2)_p-$, $-(CH_2)_nS(CH_2)_p-$, $-(CH_2)_n$alkenyl$(CH_2)_p-$, $-(CH_2)_n$alkynyl$(CH_2)_p-$, $-O(CH_2)_n-$, $-NR_2(CH_2)_n-$, or $-S(CH_2)_n-$;

X_1 and X_2 can be selected, independently, from $-N(R_8)-$, -O-, -S-, -Se-, -N=N-, -ON=CH-, $-(R_8)N-N(R_8)-$, $-ON(R_8)-$, a heterocycle, or a direct bond between L and Y_1 or Y_2, respectively;

Y_1 and Y_2 can be selected, independently, from -C(=O)-, -C(=S)-, $-S(O_2)-$, -S(O)-, -C(=NCN)-, $-P(=O)(OR_2)-$, a heteroaromatic group, or a direct bond between X_1 and Z_1 or X_2 and Z_2, respectively;

Z_1 and Z_2 can be selected, independently, from -N(R_8)-, -O-, -S-, -Se-, -N=N-, -ON=CH-, -R_8N-NR_8-, -ONR_8-, a heterocycle, or a direct bond between Y_1 or Y_2, respectively, and L;

R_8, independently for each occurrence, represents H, lower alkyl, -$(CH_2)_n$aryl (e.g., substituted or unsubstituted), -$(CH_2)_n$heteroaryl (e.g., substituted or unsubstituted), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X_1 and Z_1 or X_2 and Z_1, which ring may include one or more carbonyls;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurrence, represents an integer from 0 to 10, preferably from 0 to 5.

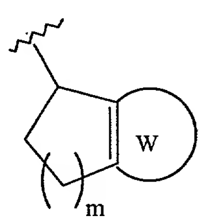
In certain embodiments, R_1 represents a substituted or unsubstituted heteroaryl group.

In certain embodiments, X_1 and X_2 can be selected from -N(R_8)-, -O-, -S-, a direct bond, and a heterocycle, Y_1 and Y_2 can be selected from -C(=O)-, -C(=S)-, and -S(O_2)-, and Z_1 or Z_2 can be selected from -N(R_8)-, -O-, -S-, a direct bond, and a heterocycle.

In certain related embodiments, X_1-Y_1-Z_1 or X_2-Y_2-Z_2 taken together represents a urea (N-C(O)-N) or an amide (N-C(O) or C(O)-N).

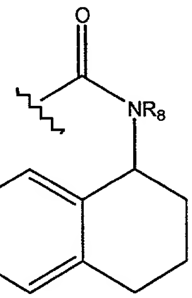
In certain embodiments, X_1 or X_2 represents a diazacarbocycle, such as a piperazine.

In certain embodiments, R_1 represents a fused cycloalkyl-aryl or cycloalkyl-heteroaryl system, for example:

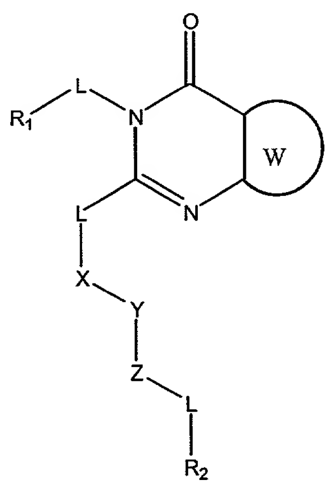


wherein W is a substituted or unsubstituted aryl or heteroaryl ring fused to the cycloalkyl ring and m is an integer from 1-4 inclusive, e.g., from 1-3, or from 1-2. The fused system may be

bound to L from any carbon of the fused system, including the position depicted above. In certain embodiments, R_1 may represent a tetrahydronaphthyl group, and preferably Y_1-X_1-L-R_1 taken together represent a tetrahydronaphthyl amide group, such as:



In embodiments wherein Y_1 and Z_1 are absent and X_1 comprises a pyrimidone, compounds useful in the present invention may be represented by general formula (XVI):



Formula XVI

wherein, as valence and stability permit,

R_1 and R_2, independently for each occurrence, represent H, lower alkyl, $-(CH_2)_n$aryl (e.g., substituted or unsubstituted), or $-(CH_2)_n$heteroaryl (e.g., substituted or unsubstituted);

L, independently for each occurrence, is absent or represents $-(CH_2)_n$-alkyl, -alkenyl-, -alkynyl-, $-(CH_2)_n$alkenyl-, $-(CH_2)_n$alkynyl-, $-(CH_2)_nO(CH_2)_p$-, $-(CH_2)_nNR_2(CH_2)_p$-, -

$(CH_2)_nS(CH_2)_p$-, -$(CH_2)_n$alkenyl$(CH_2)_p$-, -$(CH_2)_n$alkynyl$(CH_2)_p$-, -$O(CH_2)_n$-, -$NR_2(CH_2)_n$-, or -$S(CH_2)_n$-;

X can be selected from -N(R_8)-, -O-, -S-, -Se-, -N=N-, -ON=CH-, -(R_8)N-N(R_8)-, -ON(R_8)-, a heterocycle, or a direct bond between L and Y;

Y can be selected from -C(=O)-, -C(=S)-, -S(O_2)-, -S(O)-, -C(=NCN)-, -P(=O)(OR_2)-, a heteroaromatic group, or a direct bond between X and Z;

Z can be selected from -N(R_8)-, -O-, -S-, -Se-, -N=N-, -ON=CH-, -R_8N-NR_8-, -ONR_8-, a heterocycle, or a direct bond between Y and L;

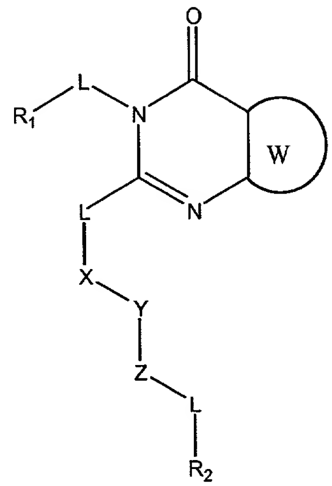
R_8, independently for each occurrence, represents H, lower alkyl, -$(CH_2)_n$aryl (e.g., substituted or unsubstituted), -$(CH_2)_n$heteroaryl (e.g., substituted or unsubstituted), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X and Z, which ring may include one or more carbonyls;

W represents a substituted or unsubstituted aryl or heteroaryl ring fused to the pyrimidone ring;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurrence, represents an integer from 0 to 10, preferably from 0 to 5.

In embodiments wherein Y_1 and Z_1 are absent and X_1 comprises a pyrimidone, compounds useful in the present invention may be represented by general formula (XVII):



Formula XVII

wherein, as valence and stability permit,

R_1 and R_2, independently for each occurrence, represent H, lower alkyl, aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$aryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$heteroaralkyl-);

L, independently for each occurrence, is absent or represents -$(CH_2)_n$-alkyl, -alkenyl-, -alkynyl-, -$(CH_2)_n$alkenyl-, -$(CH_2)_n$alkynyl-, -$(CH_2)_nO(CH_2)_p$-, -$(CH_2)_nNR_2(CH_2)_p$-, -$(CH_2)_nS(CH_2)_p$-, -$(CH_2)_n$alkenyl$(CH_2)_p$-, -$(CH_2)_n$alkynyl$(CH_2)_p$-, -$O(CH_2)_n$-, -$NR_2(CH_2)_n$-, or -$S(CH_2)_n$-, which may optionally be substituted with a group selected from H, substituted or unsubstituted lower alkyl, alkenyl, or alkynyl, cycloalkylalkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$cycloalkyl), (e.g., substituted or unsubstituted), aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$aryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$heteroaralkyl-), preferably from H, lower alkyl, -$(CH_2)_n$aryl (e.g., substituted or unsubstituted), or -$(CH_2)_n$heteroaryl (e.g., substituted or unsubstituted);

X can be selected from -$N(R_8)$-, -O-, -S-, -Se-, -N=N-, -ON=CH-, -$(R_8)N$-$N(R_8)$-, -$ON(R_8)$-, a heterocycle, or a direct bond between L and Y;

Y can be selected from -C(=O)-, -C(=S)-, -$S(O_2)$-, -S(O)-, -C(=NCN)-, -P(=O)(OR_2)-, a heteroaromatic group, or a direct bond between X and Z;

Z can be selected from -N(R_8)-, -O-, -S-, -Se-, -N=N-, -ON=CH-, -R_8N-NR_8-, -ONR_8-, a heterocycle, or a direct bond between Y and L;

R_8, independently for each occurrence, represents H, lower alkyl, aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$aryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$heteroaralkyl-), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X and Z, which ring may include one or more carbonyls;

W represents a substituted or unsubstituted aryl or heteroaryl ring fused to the pyrimidone ring;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurrence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, R_1 represents a substituted or unsubstituted aryl or heteroaryl group, e.g., a phenyl ring, a pyridine ring, etc. In certain embodiments wherein -LR_1 represents a substituted aryl or heteroaryl group, R_1 is preferably not substituted with an isopropoxy (Me_2CHO-) group. In certain embodiments wherein -LR_1 represents a substituted aryl or heteroaryl group, R_1 is preferably not substituted with an ether group. In certain embodiments, substituents on R_1 (e.g., other than hydrogen) are selected from halogen, cyano, alkyl, alkenyl, alkynyl, aryl, hydroxyl, (unbranched alkyl-O-), silyloxy, amino, nitro, thiol, amino, imino, amido, phosphoryl, phosphonate, phosphine, carbonyl, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, sulfoxide, selenoether, ketone, aldehyde, ester, or -$(CH_2)_m$-R_8. In certain embodiments, non-hydrogen substituents are selected from halogen, cyano, alkyl, alkenyl, alkynyl, aryl, nitro, thiol, imino, amido, carbonyl, carboxyl, anhydride, thioether, alkylsulfonyl, arylsulfonyl, ketone, aldehyde, and ester. In certain embodiments, non-

hydrogen substituents are selected from halogen, cyano, alkyl, alkenyl, alkynyl, nitro, amido, carboxyl, anhydride, alkylsulfonyl, ketone, aldehyde, and ester.

In certain embodiments, X can be selected from -N(R_8)-, -O-, -S-, a direct bond, and a heterocycle, Y can be selected from -C(=O)-, -C(=S)-, and -S(O_2)-, and Z can be selected from -N(R_8)-, -O-, -S-, a direct bond, and a heterocycle. In certain such embodiments, at least one of Z and X is present.

In certain related embodiments, X-Y-Z taken together represents a urea (NC(O)N) or an amide (NC(O) or C(O)N).

In certain embodiments, W is a substituted or unsubstituted benzene ring.

In certain embodiments, X represents a diazacarbocycle, such as a piperazine, e.g., substituted or unsubstituted.

In certain embodiments, X can be selected from -N(R_8)-, -O-, -S-, and a direct bond, Y can be selected from -C(=O)-, -C(=S)-, and -S(O_2)-, and Z can be selected from -N(R_8)-, -O-, -S-, and a direct bond, such that at least one of X and Z is present.

In certain embodiments R_8 represents H, lower alkyl, aralkyl, heteroaralkyl, aryl, or heteroaryl, e.g., H or lower alkyl.

In certain embodiments, X represents -NH-.

In certain embodiments, -L-X- represents -(unbranched lower alkyl)-NH-, e.g., -CH_2-NH-, -CH_2CH_2-NH-, etc.

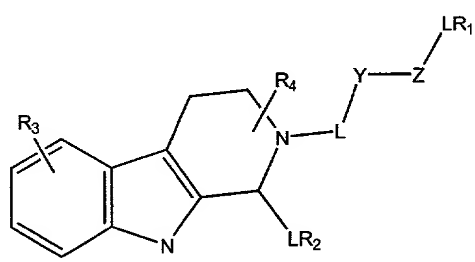
In certain embodiments, the subject antagonists can be chosen on the basis of their selectively for the *hedgehog* pathway. This selectivity can be for the *hedgehog* pathway versus other pathways, or for selectivity between particular *hedgehog* pathways, e.g., e.g., *ptc*-1, *ptc*-2, etc.

In certain preferred embodiments, the subject inhibitors inhibit *hedgehog*-mediated signal transduction with an ED_{50} of 1 mM or less, more preferably of 1 μM or less, and even more preferably of 1 nM or less.

In particular embodiments, the small molecule is chosen for use because it is more selective for one *patched* isoform over the next, e.g., 10 fold, and more preferably at least 100 or even 1000 fold more selective for one *patched* pathway (*ptc*-1, *ptc*-2) over another.

In certain embodiments, a compound which is an antagonist of the *hedgehog* pathway is chosen to selectively antagonize *hedgehog* activity over protein kinases other than PKA, such as PKC, e.g., the compound modulates the activity of the *hedgehog* pathway at least an order of magnitude more strongly than it modulates the activity of another protein kinase, preferably at least two orders of magnitude more strongly, even more preferably at least three orders of magnitude more strongly. Thus, for example, a preferred inhibitor of the *hedgehog* pathway may inhibit *hedgehog* activity with a K_i at least an order of magnitude lower than its K_i for inhibition of PKC, preferably at least two orders of magnitude lower, even more preferably at least three orders of magnitude lower. In certain embodiments, the K_i for PKA inhibition is less than 10 nM, preferably less than 1 nM, even more preferably less than 0.1 nM.

In certain embodiments, compounds useful in the present invention may be represented by general formula (IV):



Formula XVIII

wherein, as valence and stability permit,

R_1 and R_2, independently for each occurrence, represent H, substituted or unsubstituted lower alkyl, alkenyl, or alkynyl, $-(CH_2)_n$cycloalkyl (e.g., substituted or unsubstituted), $-(CH_2)_n$aryl (e.g., substituted or unsubstituted), or $-(CH_2)_n$heterocyclyl (e.g., substituted or unsubstituted);

L, independently for each occurrence, is absent or represents $-(CH_2)_n$-alkyl, -alkenyl-, -alkynyl-, $-(CH_2)_n$alkenyl-, $-(CH_2)_n$alkynyl-, $-(CH_2)_nO(CH_2)_p-$, $-(CH_2)_nNR_2(CH_2)_p-$, $-(CH_2)_nS(CH_2)_p-$, $-(CH_2)_n$alkenyl$(CH_2)_p-$, $-(CH_2)_n$alkynyl$(CH_2)_p-$, $-O(CH_2)_n-$, $-NR_2(CH_2)_n-$, or $-S(CH_2)_n-$;

X and Z, independently, can be selected from -CH-, -N(R_8)-, -O-, -S-, or -Se-;

Y can be selected from -C(=O)-, -C(=S)-, -S(O_2)-, -S(O)-, -C(=NCN)-, or -P(=O)(OR_2)-;

R_8, independently for each occurrence, represents H, substituted or unsubstituted lower alkyl, -$(CH_2)_n$cycloalkyl (e.g., substituted or unsubstituted), -$(CH_2)_n$aryl (e.g., substituted or unsubstituted), -$(CH_2)_n$heterocyclyl (e.g., substituted or unsubstituted), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X_1 and Z_1 or X_2 and Z_1, which ring may include one or more carbonyls;

R_3 and R_4, independently represent from 1-4 substituents on the ring to which they are attached, selected from, independently for each occurrence, hydrogen, halogens, alkyls, alkenyls, alkynyls, aryls, hydroxyl, =O, =S, alkoxyl, silyloxy, amino, nitro, thiol, amines, imines, amides, phosphoryls, phosphonates, phosphines, carbonyls, carboxyls, carboxamides, anhydrides, silyls, ethers, thioethers, alkylsulfonyls, arylsulfonyls, selenoethers, ketones, aldehydes, esters, or -$(CH_2)_m$-R_8;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurrence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, R_1 and R_2 are independently selected from substituted or unsubstituted aryl, heterocyclyl, branched or unbranched alkyl, or cycloalkyl. In embodiments wherein R_1 or R_2 is aryl or heterocyclyl, substituents are preferably selected from H, alkyl, acyl, carboxy, ester, amide, cyano, ether, thioether, amino, halogen, nitro, and trihalomethyl.

In certain embodiments, R_3 is absent or represents one or two substituents selected from alkyl, acyl, carboxy, ester, amide, cyano, ether, thioether, amino, acyl, halogen, nitro, and trihalomethyl.

In certain embodiments, R_4 is absent or represents one or two substituents selected from ether, amino, thioether, alkyl, aryl, (=O), or carbonyl (e.g., carboxy, ester, ketone, aldehyde, etc.).

In certain embodiments, L is absent for each occurrence, or represents $-CH_2-$ or $-CH_2CH_2-$.

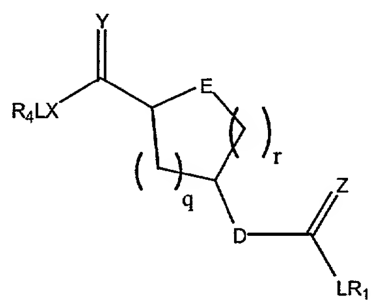
In certain embodiments, X represents NR_8. R_8 preferably represents H.

In certain embodiments, Z represents NR_8. R_8 preferably represents H.

In certain embodiments, Y represents -C(=O)-, -C(=S)-, or $-S(O_2)-$.

Exemplary compounds part 4:

As described in further detail below, it is contemplated that the subject methods can be carried out using a variety of different small molecules which can be readily identified, for example, by such drug screening assays as described herein. For example, compounds useful in the subject methods include compounds may be represented by general formula (XVIII):



Formula XVIII

wherein, as valence and stability permit,

R_1, R_2, R_3, and R_4, independently for each occurrence, represent H, lower alkyl, $-(CH_2)_n$aryl (e.g., substituted or unsubstituted), or $-(CH_2)_n$heteroaryl (e.g., substituted or unsubstituted);

L, independently for each occurrence, is absent or represents $-(CH_2)_n-$, -alkenyl-, -alkynyl-, $-(CH_2)_n$alkenyl-, $-(CH_2)_n$alkynyl-, $-(CH_2)_nO(CH_2)_p-$, $-(CH_2)_nNR_8(CH_2)_p-$, $-(CH_2)_nS(CH_2)_p-$, $-(CH_2)_n$alkenyl$(CH_2)_p-$, $-(CH_2)_n$alkynyl$(CH_2)_p-$, $-O(CH_2)_n-$, $-NR_8(CH_2)_n-$, or $-S(CH_2)_n-$;

X and D, independently, can be selected from $-N(R_8)-$, $-O-$, $-S-$, $-(R_8)N-N(R_8)-$, $-ON(R_8)-$, or a direct bond;

Y and Z, independently, can be selected from O or S;

E represents O, S, or NR_5, wherein R_5 represents LR_8 or $-(C{=}O)LR_8$.

R_8, independently for each occurrence, represents H, lower alkyl, $-(CH_2)_n$aryl (e.g., substituted or unsubstituted), $-(CH_2)_n$heteroaryl (e.g., substituted or unsubstituted), or two R_8 taken together may form a 4- to 8-membered ring;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3;

n, individually for each occurrence, represents an integer from 0 to 10, preferably from 0 to 5; and

q and r represent, independently for each occurrence, an integer from 0-2.

In certain embodiments, D does not represent N-lower alkyl. In certain embodiments, D represents an aralkyl- or heteroaralkyl-substituted amine.

In certain embodiments, R_1 represents a lower alkyl group, such as a branched alkyl, a cycloalkyl, or a cycloalkylalkyl, for example, cyclopropyl, cyclopropylmethyl, neopentyl, cyclobutyl, isobutyl, isopropyl, sec-butyl, cyclobutylmethyl, etc.

In certain embodiments, Y and Z are O.

In certain embodiments, the sum of q and r is less than 4, e.g., is 2 or 3.

In certain embodiments, XLR_4, taken together, include a cyclic amine, such as a piperazine, a morpholine, a piperidine, a pyrrolidine, etc.

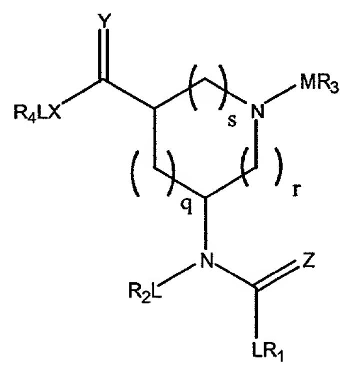
In certain embodiments, at least one of R_1, R_2, and R_3 includes an aryl or heteroaryl group. In certain related embodiments, at least two of R_1, R_2, and R_3 include an aryl or heteroaryl group. In certain embodiments, R_1 is lower alkyl.

In certain embodiments, L attached to R_1 represents O, S, or NR_8, such as NH.

In certain embodiments, E is NR_8. In certain embodiments, E represents an aralkyl- or heteroaralkyl-substituted amine, e.g., including polycyclic R_8.

In certain embodiments, X is not NH. In certain embodiments, X is included in a ring, or, taken together with –C(=Y)-, represents a tertiary amide.

In certain embodiments, compounds useful in the present invention may be represented by general formula (XIX):



Formula XIX

wherein, as valence and stability permit,

R_1, R_2, R_3, R_4, R_8, L, X, Y, Z, n, p, q, and r are as defined above;

M is absent or represents L, $-SO_2L-$, or -(C=O)L-; and

s represents, independently for each occurrence, an integer from 0-2.

In certain embodiments, Y and Z are O.

In certain embodiments, R_1 represents a lower alkyl group, such as a branched alkyl, a cycloalkyl, or a cycloalkylalkyl, for example, cyclopropyl, cyclopropylmethyl, neopentyl, cyclobutyl, isobutyl, isopropyl, sec-butyl, cyclobutylmethyl, etc.

In certain embodiments, the sum of q, r, and s is less than 5, e.g., is 2, 3, or 4.

In certain embodiments, XLR_4, taken together, include a cyclic amine, such as a piperazine, a morpholine, a piperidine, a pyrrolidine, etc.

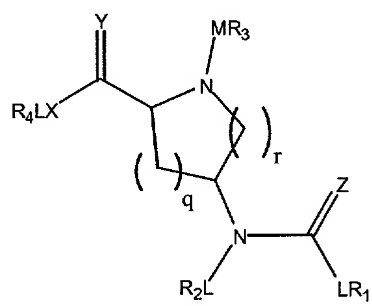
In certain embodiments, L attached to R_1 represents O, S, or NR_8, such as NH.

In certain embodiments, at least one of R_1, R_2, and R_3 includes an aryl or heteroaryl group. In certain related embodiments, at least two of R_1, R_2, and R_3 include an aryl or heteroaryl group.

In certain embodiments, M is absent.

In certain embodiments, X is not NH. In certain embodiments, X is included in a ring, or, taken together with –C(=Y)-, represents a tertiary amide.

In certain embodiments, compounds useful in the present invention may be represented by general formula (XX):



Formula XX

wherein, as valence and stability permit,

R_1, R_2, R_3, R_4, R_8, L, M, X, Y, Z, n, p, q, and r are as defined above.

In certain embodiments, Y and Z are O.

In certain embodiments, R_1 represents a lower alkyl group, preferably a branched alkyl, a cycloalkyl, or a cycloalkylalkyl, for example, cyclopropyl, cyclopropylmethyl, neopentyl, cyclobutyl, isobutyl, isopropyl, sec-butyl, cyclobutylmethyl, etc.

In certain embodiments, the sum of q and r is less than 4, e.g., is 2 or 3.

In certain embodiments, XLR_4, taken together, include a cyclic amine, such as a piperazine, a morpholine, a piperidine, a pyrrolidine, etc.

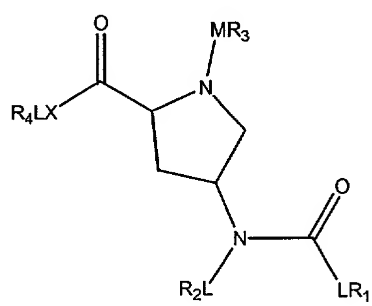
In certain embodiments, at least one of R_1, R_2, and R_3 includes an aryl or heteroaryl group. In certain related embodiments, at least two of R_1, R_2, and R_3 include an aryl or heteroaryl group. In certain embodiments, R_1 is lower alkyl.

In certain embodiments, L attached to R_1 represents O, S, or NR_8, such as NH.

In certain embodiments, M is absent.

In certain embodiments, X is not NH. In certain embodiments, X is included in a ring, or, taken together with –C(=Y)-, represents a tertiary amide.

In certain embodiments, compounds useful in the present invention may be represented by general formula (XXI):



Formula XXI

wherein, as valence and stability permit,

R_1, R_2, R_3, R_4, R_8, L, M, X, n, and p are as defined above.

In certain embodiments, XLR_4, taken together, include a cyclic amine, such as a piperazine, a morpholine, a piperidine, a pyrrolidine, etc.

In certain embodiments, R_1 represents a lower alkyl group, preferably a branched alkyl, a cycloalkyl, or a cycloalkylalkyl, for example, cyclopropyl, cyclopropylmethyl, neopentyl, cyclobutyl, isobutyl, isopropyl, sec-butyl, cyclobutylmethyl, etc.

In certain embodiments, at least one of R_1, R_2, and R_3 includes an aryl or heteroaryl group. In certain related embodiments, at least two of R_1, R_2, and R_3 include an aryl or heteroaryl group. In certain embodiments, R_1 is lower alkyl.

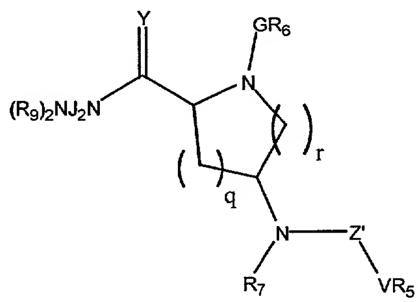
In certain embodiments, L attached to R_1 represents O, S, or NR_8, such as NH.

In certain embodiments, M is absent.

In certain embodiments, X is not NH. In certain embodiments, X is included in a ring, or, taken together with –C(=Y)-, represents a tertiary amide.

In certain embodiments L represents a direct bond for all occurrences.

In certain embodiments, compounds useful in the present invention may be represented by general formula (XXII):



Formula XXII

wherein, as valence and stability permit,

Y, n, p, q, and r are as defined above;

Z' represents -C(=O)-, -C(=S)-, -C(=NH)-, SO_2, or SO, preferably -C(=O)-, -C(=S)-;

V is absent or represents O, S, or NR_8;

G is absent or represents –C(=O)- or –SO_2-;

J, independently for each occurrence, represents H or substituted or unsubstituted lower alkyl or alkylene, such as methyl, ethyl, methylene, ethylene, etc., attached to NC(=Y), such that both occurrences of N adjacent to J are linked through at least one occurrence of J, and

R_9, independently for each occurrence, is absent or represents H or lower alkyl, or two occurrences of J or one occurrence of J taken together with one occurrence of R_9, forms a ring of from 5 to 7 members, which ring includes one or both occurrences of N;

R_5 represents substituted or unsubstituted alkyl (e.g., branched or unbranched), alkenyl (e.g., branched or unbranched), alkynyl (e.g., branched or unbranched), cycloalkyl, or cycloalkylalkyl;

R_6 represents substituted or unsubstituted aryl, aralkyl, heteroaryl, heteroaralkyl, heterocyclyl, heterocyclylalkyl, cycloalkyl, or cycloalkylalkyl, including polycyclic groups; and

R_7 represents substituted or unsubstituted aryl, aralkyl, heteroaryl, or heteroaralkyl.

In certain embodiments, Y is O. In certain embodiments, Z' represents SO_2, -C(=O)-, or -C(=S)-.

In certain embodiments, the sum of q and r is less than 4.

In certain embodiments, NJ_2N, taken together, represent a cyclic diamine, such as a piperazine, etc., which may be substituted or unsubstituted, e.g., with one or more substitutents such as oxo, lower alkyl, lower alkyl ether, etc. In certain other embodiments, NJ_2 or NJR_9 taken together represent a substituted or unsubstituted heterocyclic ring to which the other occurrence of N is attached. In certain embodiments, one or both occurrences of J are substituted with one or more of lower alkyl, lower alkyl ether, lower alkyl thioether, amido, oxo, etc. In certain embodiments, a heterocyclic ring that comprises an occurrence of J has from 5 to 8 members.

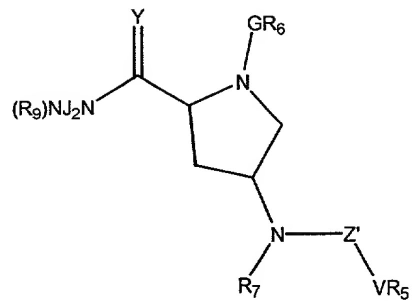
In certain embodiments, R_5 represents a branched alkyl, cycloalkyl, or cycloalkylalkyl.

In certain embodiments, R_6 includes at least one heterocyclic ring, such as a thiophene, furan, oxazole, benzodioxane, benzodioxole, pyrrole, indole, etc.

In certain embodiments, R_7 represents a phenyl alkyl, such as a benzyl group, optionally substituted with halogen, hydroxyl, lower alkyl, nitro, cyano, lower alkyl ether (e.g., optionally substituted, such as CHF_2CF_2O), or lower alkyl thioether (e.g., optionally substituted, such as CF_3S).

In certain embodiments, R_8, when it occurs in V, represents H or lower alkyl, preferably H.

In certain embodiments, compounds useful in the present invention may be represented by general formula (XXIII):



Formula XXIII

wherein, as valence and stability permit,

R_5, R_6, R_7, R_8, R_9, R_{10}, G, J, V, Y, Z', n, and p are as defined above.

In certain embodiments, Y is O. In certain embodiments, Z' represents SO_2, -C(=O)-, or -C(=S)-.

In certain embodiments, NJ_2N, taken together, represent a heterocyclic ring, such as a piperazine, etc., which may be substituted or unsubstituted, e.g., with one or more substitutents such as oxo, lower alkyl, lower alkyl ether, etc. In certain other embodiments, NJ_2 or NJR_9 taken together represent a substituted or unsubstituted heterocyclic ring to which the other occurrence of N is attached. In certain embodiments, one or both occurrences of J are substituted with one or more of lower alkyl, lower alkyl ether, lower alkyl thioether, amido, oxo, etc. In certain embodiments, a heterocyclic ring that comprises an occurrence of J has from 5 to 8 members.

In certain embodiments, R_5 represents a branched alkyl, cycloalkyl, or cycloalkylalkyl.

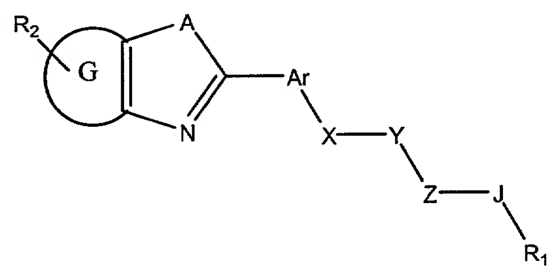
In certain embodiments, R_6 includes at least one heterocyclic ring, such as a thiophene, furan, oxazole, benzodioxane, benzodioxole, pyrrole, indole, etc.

In certain embodiments, R_7 represents a phenyl alkyl, such as a benzyl group, optionally substituted with halogen, hydroxyl, lower alkyl, nitro, cyano, lower alkyl ether (e.g., optionally substituted, such as CHF_2CF_2O), or lower alkyl thioether (e.g., optionally substituted, such as CF_3S).

In certain embodiments, R_8, when it occurs in V, represents H or lower alkyl, preferably H.

Exemplary compounds part 5:

As described in further detail below, it is contemplated that the subject methods can be carried out using a variety of different small molecules which can be readily identified, for example, by such drug screening assays as described herein. For example, compounds useful in the subject methods include compounds may be represented by general formula (XXIV):



Formula XXIV

wherein, as valence and stability permit,

X and Z, independently, represent -N(R_7)-, -O-, -S-, -(R_7)N-N(R_7)-, -ON(R_7)-, or a direct bond, preferably -N(R_7)-, -O-, -S-, or a direct bond;

Y represents -C(=O)-, -C(=S)-, -C(=NR_7)-, SO_2, or SO, preferably -C(=O)-, SO_2, or -C(=S)-;

A represents O, S, or NR_7, preferably O or NH, and most preferably NH;

G represents a cycloalkyl, heterocyclyl, aryl, or heteroaryl ring fused to the ring to which it is attached, preferably an aryl or heteroaryl ring.

Ar represents a substituted or unsubstituted aryl or heteroaryl ring, such as a substituted or unsubstituted phenyl ring;

R_1 represents H or substituted or unsubstituted alkyl, alkenyl, alkynyl, aryl, heteroaryl, heterocyclyl, or cycloalkyl, including polycyclic groups;

R_2 represents from 0-4 substituents on the ring to which it is attached, such as halogen, lower alkyl, lower alkenyl, aryl, heteroaryl, carbonyl group (e.g., ester, carboxyl, or formyl), thiocarbonyl (e.g., thioester, thiocarboxylate, or thioformate), ketone, aldehyde, amino, acylamino, amido, amidino, cyano, nitro, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, J-R_8, J-OH, J-lower alkyl, J-lower alkenyl, J-R_8, J-SH, J-NH_2, protected forms of the above, or any two R_2, when occurring

more than once in a cyclic or polycyclic structure, can be taken together form a 4- to 8-membered cycloalkyl, aryl, or heteroaryl;

R_7, independently for each occurrence, represents H, lower alkyl (e.g., substituted or unsubstituted), J-cycloalkyl (e.g., substituted or unsubstituted), J-heterocyclyl (e.g., substituted or unsubstituted), J-aryl (e.g., substituted or unsubstituted), J-heteroaryl (e.g., substituted or unsubstituted);

R_8, independently for each occurrence, represents H, lower alkyl (e.g., substituted or unsubstituted), cycloalkyl (e.g., substituted or unsubstituted), heterocyclyl (e.g., substituted or unsubstituted), aryl (e.g., substituted or unsubstituted), or heteroaryl (e.g., substituted or unsubstituted); and

J represents, independently for each occurrence, a chain having from 0-8 (preferably from 0-4) units selected from CK_2, NK, O, and S, wherein K represents, independently for each occurrence, H or lower alkyl.

In certain embodiments, at least one of Z and X is not a direct bond. In certain embodiments, X-Y-Z includes an amide, urea, or sulfonamide. In certain embodiments, X is selected from -N(R_8)-, -O-, -S-, and preferably represents NH.

In certain embodiments, R_1 includes an aryl or heteroaryl ring, optionally substituted with from 1-5 substituents, such as nitro, halogen, cyano, lower alkyl, acylamino (e.g., R_8-C(=O)NH-), alkoxy, alkylamino, a substituted or unsubstituted cycloalkyl, heterocyclyl, aryl, or heteroaryl fused to the aryl or heteroaryl ring.

In certain embodiments, X and the ring comprising A are disposed on Ar in a meta (i.e., 1,3) relationship.

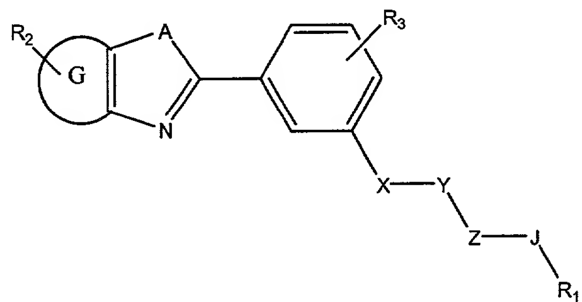
In certain embodiments, G represents a phenyl or piperidine ring.

In certain embodiments, J is absent.

In certain embodiments, R_2 represents from 1-4 substituents selected from halogen, cyano, nitro, alkoxy, amino, acylamino (e.g., R_8-C(=O)NH-), a substituted or unsubstituted

cycloalkyl, heterocyclyl, aryl, or heteroaryl fused to G, and substituted or unsubstituted lower alkyl.

In certain embodiments, compounds useful in the present invention may be represented by general formula (XXV):



Formula XXV

wherein, as valence and stability permit,

X and Z, independently, represent -N(R_7)-, -O-, -S-, -(R_7)N-N(R_7)-, -ON(R_7)-, or a direct bond, preferably -N(R_7)-, -O-, -S-, or a direct bond;

Y represents -C(=O)-, -C(=S)-, -C(=NR_7)-, SO_2, or SO, preferably -C(=O)-, SO_2, or -C(=S)-;

A represents O, S, or NR_7, preferably O or NH, and most preferably NH;

G represents a cycloalkyl, heterocyclyl, aryl, or heteroaryl ring fused to the ring to which it is attached, preferably an aryl or heteroaryl ring.

R_1 represents H or substituted or unsubstituted alkyl, alkenyl, alkynyl, aryl, heteroaryl, heterocyclyl, or cycloalkyl, including polycyclic groups;

R_2 represents from 0-4 substituents on the ring to which it is attached, such as halogen, lower alkyl, lower alkenyl, aryl, heteroaryl, carbonyl group (e.g., ester, carboxyl, or formyl), thiocarbonyl (e.g., thioester, thiocarboxylate, or thioformate), ketone, aldehyde, amino, acylamino, amido, amidino, cyano, nitro, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, J-R_8, J-OH, J-lower alkyl, J-lower alkenyl, J-R_8, J-SH, J-NH_2, protected forms of the above, or any two R_2, when occurring

more than once in a cyclic or polycyclic structure, can be taken together form a 4- to 8-membered cycloalkyl, aryl, or heteroaryl;

R_3 represents from 0-4 substituents on the ring to which it is attached, such as halogen, hydroxyl, alkoxy, amino, alkylamino, cyano, nitro, substituted or unsubstituted lower alkyl, and acyl, preferably halogen or substituted or unsubstituted lower alkyl;

R_7, independently for each occurrence, represents H, lower alkyl (e.g., substituted or unsubstituted), J-cycloalkyl (e.g., substituted or unsubstituted), J-heterocyclyl (e.g., substituted or unsubstituted), J-aryl (e.g., substituted or unsubstituted), J-heteroaryl (e.g., substituted or unsubstituted);

R_8, independently for each occurrence, represents H, lower alkyl (e.g., substituted or unsubstituted), cycloalkyl (e.g., substituted or unsubstituted), heterocyclyl (e.g., substituted or unsubstituted), aryl (e.g., substituted or unsubstituted), or heteroaryl (e.g., substituted or unsubstituted); and

J represents, independently for each occurrence, a chain having from 0-8 (preferably from 0-4) units selected from CK_2, NK, O, and S, wherein K represents, independently for each occurrence, H or lower alkyl.

In certain embodiments, at least one of Z and X is not a direct bond. In certain embodiments, X-Y-Z includes an amide, urea, or sulfonamide. In certain embodiments, X is selected from -N(R_8)-, -O-, -S-, and preferably represents NH.

In certain embodiments, R_1 includes an aryl or heteroaryl ring, optionally substituted with from 1-5 substituents, such as nitro, halogen, cyano, lower alkyl, acylamino (e.g., R_8-C(=O)NH-), alkoxy, alkylamino, a substituted or unsubstituted cycloalkyl, heterocyclyl, aryl, or heteroaryl fused to the aryl or heteroaryl ring.

In certain embodiments, G represents a phenyl or piperidine ring.

In certain embodiments, J is absent.

In certain embodiments, R_2 represents from 1-4 substituents selected from halogen, cyano, nitro, alkoxy, amino, acylamino (e.g., R_8-C(=O)NH-), a substituted or unsubstituted

cycloalkyl, heterocyclyl, aryl, or heteroaryl fused to G, and substituted or unsubstituted lower alkyl.

In certain embodiments, R_3 includes a substituent, such as a substituted or unsubstituted alkyl or a halogen, at a position para either to X or to the ring including A.

In certain embodiments, the subject antagonists can be chosen on the basis of their selectivity for the *hedgehog* pathway. This selectivity can be for the *hedgehog* pathway versus other pathways, or for selectivity between particular *hedgehog* pathways, e.g., *ptc*-1, *ptc*-2, etc.

In certain preferred embodiments, the subject inhibitors inhibit *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothened* gain-of-function mediated signal transduction with an ED_{50} of 1 mM or less, more preferably of 1 μM or less, and even more preferably of 1 nM or less. Similarly, in certain preferred embodiments, the subject inhibitors inhibit activity of the *hedgehog* pathway with a K_i less than 10 nM, preferably less than 1 nM, even more preferably less than 0.1 nM.

In particular embodiments, the small molecule is chosen for use because it is more selective for one *patched* isoform over the next, e.g., 10-fold, and more preferably at least 100- or even 1000-fold more selective for one *patched* pathway (*ptc*-1, *ptc*-2) over another.

In certain embodiments, a compound which is an antagonist of the *hedgehog* pathway is chosen to selectively antagonize *hedgehog* activity over protein kinases other than PKA, such as PKC, e.g., the compound modulates the activity of the *hedgehog* pathway at least an order of magnitude more strongly than it modulates the activity of another protein kinase, preferably at least two orders of magnitude more strongly, even more preferably at least three orders of magnitude more strongly. Thus, for example, a preferred inhibitor of the *hedgehog* pathway may inhibit *hedgehog* activity with a K_i at least an order of magnitude lower than its K_i for inhibition of PKC, preferably at least two orders of magnitude lower, even more preferably at least three orders of magnitude lower. In certain embodiments, the K_i for PKA inhibition is less than 10 nM, preferably less than 1 nM, even more preferably less than 0.1 nM.

In certain preferred embodiments, *hedgehog* antagonists include AY9944, triparanol, jervine, cyclopamine and tomatidine (see Figure 1), compound A (see Figure 2) and compound B (see Figure 3).

Antagonist *hedgehog* mutants:

It is anticipated that certain mutant forms of a *hedgehog* protein may act as *hedgehog* antagonists. While not wishing to be bound to any particular theory, it is well known that mutant forms of protein signaling factors are capable of binding to the appropriate receptor and yet not capable of activating the receptor. Such mutant proteins act as antagonists by displacing the wild-type proteins and blocking the normal receptor activation. Mutant *hedgehog* proteins may behave similarly. Alternatively, altered *hedgehog* proteins may bind directly to and inhibit the wild-type forms of *hedgehog* and so act as antagonists. There are many well known methods for obtaining mutants with a desired activity.

Antagonist forms of *hedgehog* may be identified by using a *hedgehog* sensitive screening system. For example, a cell line transfected with a *gli*-1-lacZ reporter gene construct could be monitored for beta-galactosidase activity. *Gli*-1 is a reporter for activation of the *hedgehog* signaling pathway and *hedgehog* mutants that inhibit *gli*-1-driven reporter gene expression would be *hedgehog* antagonists. Any number of reporter genes may be used, including luciferase, green fluorescent protein (and variants including yellow, red, blue and cyan), GUS, and other fluorescent or chromogenic proteins.

Methods for generating large pools of mutant proteins are well known in the art. In one embodiment, the invention contemplates using *hedgehog* polypeptides generated by combinatorial mutagenesis. Such methods, as are known in the art, are convenient for generating both point and truncation mutants, and can be especially useful for identifying potential variant sequences (e.g., homologs) that are functional in binding to a receptor for *hedgehog* proteins. The purpose of screening such combinatorial libraries is to generate, for example, novel *hedgehog* homologs that can act as either agonists or antagonists. To illustrate, *hedgehog* homologs can be engineered by the present method to provide more efficient binding to a

cognate receptor, such as *patched*, yet still retain at least a portion of an activity associated with *hedgehog*. Thus, combinatorially derived homologs can be generated to have an increased potency relative to a naturally occurring form of the protein. Likewise, *hedgehog* homologs can be generated by the present combinatorial approach to act as antagonists, in that they are able to mimic, for example, binding to other extracellular matrix components (such as receptors), yet not induce any biological response, thereby inhibiting the action of authentic *hedgehog* or *hedgehog* agonists. Moreover, manipulation of certain domains of *hedgehog* by the present method can provide domains more suitable for use in fusion proteins, such as one that incorporates portions of other proteins which are derived from the extracellular matrix and/or which bind extracellular matrix components.

To further illustrate the state of the art of combinatorial mutagenesis, it is noted that the review article of Gallop et al. (1994) J Med Chem 37:1233 describes the general state of the art of combinatorial libraries as of the earlier 1990's. In particular, Gallop et al state at page 1239 "[s]creening the analog libraries aids in determining the minimum size of the active sequence and in identifying those residues critical for binding and intolerant of substitution". In addition, the Ladner et al. PCT publication WO90/02809, the Goeddel et al. U.S. Patent 5,223,408, and the Markland et al. PCT publication WO92/15679 illustrate specific techniques which one skilled in the art could utilize to generate libraries of *hedgehog* variants which can be rapidly screened to identify variants/fragments which retained a particular activity of the *hedgehog* polypeptides. These techniques are exemplary of the art and demonstrate that large libraries of related variants/truncants can be generated and assayed to isolate particular variants without undue experimentation. Gustin et al. (1993) Virology 193:653, and Bass et al. (1990) Proteins: Structure, Function and Genetics 8:309-314 also describe other exemplary techniques from the art which can be adapted as means for generating mutagenic variants of *hedgehog* polypeptides.

Indeed, it is plain from the combinatorial mutagenesis art that large scale mutagenesis of *hedgehog* proteins, without any preconceived ideas of which residues were critical to the biological function, can generate wide arrays of variants having equivalent biological activity. Indeed, it is the ability of combinatorial techniques to screen billions of different variants by high throughout analysis that removes any requirement of a priori understanding or knowledge of critical residues.

To illustrate, the amino acid sequences for a population of *hedgehog* homologs or other related proteins are aligned, preferably to promote the highest homology possible. Such a population of variants can include, for example, *hedgehog* homologs from one or more species. Amino acids that appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In a preferred embodiment, the variegated library of *hedgehog* variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential *hedgehog* sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of *hedgehog* sequences therein.

As illustrated in PCT publication WO 95/18856, to analyze the sequences of a population of variants, the amino acid sequences of interest can be aligned relative to sequence homology. The presence or absence of amino acids from an aligned sequence of a particular variant is relative to a chosen consensus length of a reference sequence, which can be real or artificial.

In an illustrative embodiment, alignment of exons 1, 2 and a portion of exon 3 encoded sequences (e.g., the N-terminal approximately 221 residues of the mature protein) of each of the Shh clones produces a degenerate set of Shh polypeptides represented by the general formula:

C-G-P-G-R-G-X(1)-G-X(2)-R-R-H-P-K-K-L-T-P-L-A-Y-K-Q-F-I-P-N-V-A-E-K-T-L-G-A-S-G-R-Y-E-G-K-I-X(3)-R-N-S-E-R-F-K-E-L-T-P-N-Y-N-P-D-I-I-F-K-D-E-E-N-T-G-A-D-R-L-M-T-Q-R-C-K-D-K-L-N-X(4)-L-A-I-S-V-M-N-X(5)-W-P-G-V-X(6)-L-R-V-T-E-G-W-D-E-D-G-H-H-X(7)-E-E-S-L-H-Y-E-G-R-A-V-D-I-T-T-S-D-R-D-X(8)-S-K-Y-G-X(9)-L-X(10)-R-L-A-V-E-A-G-F-D-W-V-Y-Y-E-S-K-A-H-I-H-C-S-V-K-A-E-N-S-V-A-A-K-S-G-G-C-F-P-G-S-A-X(11)-V-X(12)-L-X(13)-X(14)-G-G-X(15)-K-X-(16)-V-K-D-L-X(17)-P-G-D-X(18)-V-L-A-A-D-X(19)-X(20)-G-X(21)-L-X(22)-X(23)-S-D-F-X(24)-X(25)-F-X(26)-D-R (SEQ ID No: 21

wherein each of the degenerate positions "X" can be an amino acid which occurs in that position in one of the human, mouse, chicken or zebrafish Shh clones, or, to expand the library, each X can also be selected from amongst amino acid residue which would be conservative substitutions for the amino acids which appear naturally in each of those positions. For instance, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Phe, Tyr or Trp ; Xaa(2) represents Arg, His or Lys; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(4) represents Gly, Ala, Val, Leu, Ile, Ser or

Thr; Xaa(5) represents Lys, Arg, His, Asn or Gln; Xaa(6) represents Lys, Arg or His; Xaa(7) represents Ser, Thr, Tyr, Trp or Phe; Xaa(8) represents Lys, Arg or His; Xaa(9) represents Met, Cys, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Leu, Val, Met, Thr or Ser; Xaa(12) represents His, Phe, Tyr, Ser, Thr, Met or Cys; Xaa(13) represents Gln, Asn, Glu, or Asp; Xaa(14) represents His, Phe, Tyr, Thr, Gln, Asn, Glu or Asp; Xaa(15) represents Gln, Asn, Glu, Asp, Thr, Ser, Met or Cys; Xaa(16) represents Ala, Gly, Cys, Leu, Val or Met; Xaa(17) represents Arg, Lys, Met, Ile, Asn, Asp, Glu, Gln, Ser, Thr or Cys; Xaa(18) represents Arg, Lys, Met or Ile; Xaa(19) represents Ala, Gly, Cys, Asp, Glu, Gln, Asn, Ser, Thr or Met; Xaa(20) represents Ala, Gly, Cys, Asp, Asn, Glu or Gln; Xaa(21) represents Arg, Lys, Met, Ile, Asn, Asp, Glu or Gln; Xaa(22) represent Leu, Val, Met or Ile; Xaa(23) represents Phe, Tyr, Thr, His or Trp; Xaa(24) represents Ile, Val, Leu or Met; .Xaa(25) represents Met, Cys, Ile, Leu, Val, Thr or Ser; Xaa(26) represents Leu, Val, Met, Thr or Ser. In an even more expansive library, each X can be selected from any amino acid.

In similar fashion, alignment of each of the human, mouse, chicken and zebrafish *hedgehog* clones, can provide a degenerate polypeptide sequence represented by the general formula:

C-G-P-G-R-G-X(1)-X(2)-X(3)-R-R-X(4)-X(5)-X(6)-P-K-X(7)-L-X(8)-P-L-X(9)-Y-K-Q-F-X(10)-P-X(11)-X(12)-X(13)-E-X(14)-T-L-G-A-S-G-X(15)-X(16)-E-G-X(17)-X(18)-X(19)-R-X(20)-S-E-R-F-X(21)-X(22)-L-T-P-N-Y-N-P-D-I-I-F-K-D-E-E-N-X(23)-G-A-D-R-L-M-T-X(24)-R-C-K-X(25)-X(26)-X(27)-N-X(28)-L-A-I-S-V-M-N-X(29)-W-P-G-V-X(30)-L-R-V-T-E-G-X(31)-D-E-D-G-H-H-X(32)-X(33)-X(34)-S-L-H-Y-E-G-R-A-X(35)-D-I-T-T-S-D-R-D-X(36)-X(37)-K-Y-G-X(38)-L-X(39)-R-L-A-V-E-A-G-F-D-W-V-Y-Y-E-S-X(40)-X(41)-H-X(42)-H-X(43)-S-V-K-X(44)-X(45) (SEQ IDNo:22)

wherein, as above, each of the degenerate positions "X" can be an amino acid which occurs in a corresponding position in one of the wild-type clones, and may also include amino acid residue which would be conservative substitutions, or each X can be any amino acid residue. In an exemplary embodiment, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Pro, Phe or Tyr; Xaa(2) represents Gly, Ala, Val, Leu or Ile; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Lys, His or Arg; Xaa(4) represents Lys, Arg or His; Xaa(5) represents Phe, Trp, Tyr or an amino acid gap; Xaa(6) represents Gly, Ala, Val, Leu, Ile or an amino acid gap; Xaa(7) represents Asn, Gln, His, Arg or

Lys; Xaa(8) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(9) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Ser, Thr, Gln or Asn; Xaa(12) represents Met, Cys, Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(13) represents Gly, Ala, Val, Leu, Ile or Pro; Xaa(14) represents Arg, His or Lys; Xaa(15) represents Gly, Ala, Val, Leu, Ile, Pro, Arg, His or Lys; Xaa(16) represents Gly, Ala, Val, Leu, Ile, Phe or Tyr; Xaa(17) represents Arg, His or Lys; Xaa(18) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(19) represents Thr or Ser; Xaa(20) represents Gly, Ala, Val, Leu, Ile, Asn or Gln; Xaa(21) represents Arg, His or Lys; Xaa(22) represents Asp or Glu; Xaa(23) represents Ser or Thr; Xaa(24) represents Glu, Asp, Gln or Asn; Xaa(25) represents Glu or Asp; Xaa(26) represents Arg, His or Lys; Xaa(27) represents Gly, Ala, Val, Leu or Ile; Xaa(28) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(29) represents Met, Cys, Gln, Asn, Arg, Lys or His; Xaa(30) represents Arg, His or Lys; Xaa(31) represents Trp, Phe, Tyr, Arg, His or Lys; Xaa(32) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Tyr or Phe; Xaa(33) represents Gln, Asn, Asp or Glu; Xaa(34) represents Asp or Glu; Xaa(35) represents Gly, Ala, Val, Leu, or Ile; Xaa(36) represents Arg, His or Lys; Xaa(37) represents Asn, Gln, Thr or Ser; Xaa(38) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Met or Cys; Xaa(39) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(40) represents Arg, His or Lys; Xaa(41) represents Asn, Gln, Gly, Ala, Val, Leu or Ile; Xaa(42) represents Gly, Ala, Val, Leu or Ile; Xaa(43) represents Gly, Ala, Val, Leu, Ile, Ser, Thr or Cys; Xaa(44) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; and Xaa(45) represents Asp or Glu.

There are many ways by which the library of potential *hedgehog* homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential *hedgehog* sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al. (1981) Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) Science 249:386-390; Roberts et

al. (1992) PNAS 89:2429-2433; Devlin et al. (1990) Science 249: 404-406; Cwirla et al. (1990) PNAS 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of *hedgehog* homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate *hedgehog* sequences created by combinatorial mutagenesis techniques.

In yet another screening assay, the candidate *hedgehog* gene products are displayed on the surface of a cell or viral particle, and the ability of particular cells or viral particles to associate with a *hedgehog*-binding moiety (such as the *patched* protein or other *hedgehog* receptor) via this gene product is detected in a "panning assay". Such panning steps can be carried out on cells cultured from embryos. For instance, the gene library can be cloned into the gene for a surface membrane protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al. (1991) Bio/Technology 9:1370-1371; and Goward et al. (1992) TIBS 18:136-140). In a similar fashion, fluorescently labeled molecules that bind hedgehog can be used to score for potentially functional *hedgehog* homologs. Cells can be visually inspected and separated under a fluorescence microscope, or, where the morphology of the cell permits, separated by a fluorescence-activated cell sorter.

In an alternate embodiment, the gene library is expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at very high concentrations, large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage is recovered from an affinity

matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical E. coli filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) J. Biol. Chem. 267:16007-16010; Griffths et al. (1993) EMBO J 12:725-734; Clackson et al. (1991) Nature 352:624-628; and Barbas et al. (1992) PNAS 89:4457-4461).

In an illustrative embodiment, the recombinant phage antibody system (RPAS, Pharamacia Catalog number 27-9400-01) can be easily modified for use in expressing and screening *hedgehog* combinatorial libraries. For instance, the pCANTAB 5 phagemid of the RPAS kit contains the gene that encodes the phage gIII coat protein. The *hedgehog* combinatorial gene library can be cloned into the phagemid adjacent to the gIII signal sequence such that it will be expressed as a gIII fusion protein. After ligation, the phagemid is used to transform competent E. coli TG1 cells. Transformed cells are subsequently infected with M13KO7 helper phage to rescue the phagemid and its candidate *hedgehog* gene insert. The resulting recombinant phage contain phagemid DNA encoding a specific candidate *hedgehog*, and display one or more copies of the corresponding fusion coat protein. The phage-displayed candidate *hedgehog* proteins that are capable of binding a hedgehog receptor are selected or enriched by panning. For instance, the phage library can be applied to cells that express the *patched* protein and unbound phage washed away from the cells. The bound phage is then isolated, and if the recombinant phage express at least one copy of the wild type gIII coat protein, they will retain their ability to infect E. coli. Thus, successive rounds of reinfection of E. coli, and panning will greatly enrich for *hedgehog* homologs, which can then be screened for further biological activities in order to differentiate agonists and antagonists.

Combinatorial mutagenesis has a potential to generate very large libraries of mutant proteins, e.g., in the order of 10^{26} molecules. Combinatorial libraries of this size may be technically challenging to screen even with high throughput screening assays such as phage display. To overcome this problem, a new technique has been developed recently, recursive ensemble mutagenesis (REM), which allows one to avoid the very high proportion of non-functional proteins in a random library and simply enhances the frequency of functional proteins, thus decreasing the complexity required to achieve a useful sampling of sequence space. REM is

an algorithm that enhances the frequency of functional mutants in a library when an appropriate selection or screening method is employed (Arkin and Yourvan, 1992, PNAS USA 89:7811-7815; Yourvan et al., 1992, Parallel Problem Solving from Nature, 2., In Maenner and Manderick, eds., Elsevir Publishing Co., Amsterdam, pp. 401-410; Delgrave et al., 1993, Protein Engineering 6(3):327-331).

Antibody antagonists:

It is anticipated that antibodies can act as *hedgehog* antagonists. Antibodies can have extraordinary affinity and specificity for particular epitopes. Antibodies that bind to any protein in the *hedgehog* signaling pathway may have the capacity to act as antagonists. Antibodies that bind to *hedgehog, smoothened* or *gli-1* may act by simply sterically hindering the proper protein-protein interactions or occupying active sites. Antibodies that bind to *patched* proteins may act as antagonists if they cause hyperactivation of the *patched* protein, for example stimulating *patched* association with *smoothened.* Proteins with extracellular domains are readily bound by exogenously supplied antibodies.

Antibodies with *hedgehog* antagonist activity can be identified in much the same way as other *hedgehog* antagonists. For example, candidate antibodies can be administered to cells expressing a *hedgehog* reporter gene, and antibodies that cause decreased reporter gene expression are antagonists.

In one variation, antibodies of the invention can be single chain antibodies (scFv), comprising variable antigen binding domains linked by a polypeptide linker. Single chain antibodies are expressed as a single polypeptide chain and can be expressed in bacteria and as part of a phage display library. In this way, phage that express the appropriate scFv will have *hedgehog* antagonist activity. The nucleic acid encoding the single chain antibody can then be recovered from the phage and used to produce large quantities of the scFv. Construction and screening of scFv libraries is extensively described in various publications (U.S. Patents 5,258,498; 5,482,858; 5,091,513; 4,946,778; 5,969,108; 5,871,907; 5,223,409; 5,225,539).

Antisense, ribozyme and triplex techniques:

Another aspect of the invention relates to the use of the isolated nucleic acid in "antisense" therapy. As used herein, "antisense" therapy refers to administration or in situ generation of oligonucleotide molecules or their derivatives which specifically hybridize (e.g., bind) under cellular conditions, with the cellular mRNA and/or genomic DNA encoding one or more of the subject *hedgehog* pathway proteins so as to inhibit expression of that protein, e.g., by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy that relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes a hedge*hog* signaling protein. Alternatively, the antisense construct is an oligonucleotide probe that is generated ex vivo and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of a *hedgehog* signaling gene. Such oligonucleotide probes are preferably modified oligonucleotides that are resistant to endogenous nucleases, e.g., exonucleases and/or endonucleases, and are therefore stable in vivo. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) BioTechniques 6:958-976; and Stein et al. (1988) Cancer Res 48:2659- 2668. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between the -10 and +10 regions of the *hedgehog* signaling gene nucleotide sequence of interest, are preferred.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to mRNA encoding a *hedgehog* signaling protein. The antisense oligonucleotides will bind to the mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. In the case of double-stranded antisense

nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the mRNA, e.g., the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have recently been shown to be effective at inhibiting translation of mRNAs as well. (Wagner, R. 1994. Nature 372:333). Therefore, oligonucleotides complementary to either the 5' or 3' untranslated, non-coding regions of a gene could be used in an antisense approach to inhibit translation of that mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could also be used in accordance with the invention. Whether designed to hybridize to the 5', 3' or coding region of mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably less that about 100 and more preferably less than about 50, 25, 17 or 10 nucleotides in length.

Regardless of the choice of target sequence, it is preferred that in vitro studies are first performed to quantitate the ability of the antisense oligonucleotide to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the target RNA or protein with that of an internal control RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors), or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. W088/09810, published December 15, 1988) or the blood- brain barrier (see, e.g., PCT Publication No. W089/10134, published April 25, 1988), hybridization-triggered cleavage agents. (See, e.g., Krol et al., 1988, BioTechniques 6:958- 976) or intercalating agents. (See, e.g., Zon, 1988, Pharm. Res. 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5- bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5- (carboxyhydroxytriethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5- carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6- isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D- mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6- isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5- oxyacetic acid methyl ester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3- N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

The antisense oligonucleotide can also contain a neutral peptide-like backbone. Such molecules are termed peptide nucleic acid (PNA)-oligomers and are described, e.g., in Perry-

O'Keefe et al. (1996) Proc. Natl. Acad. Sci. U.S.A. 93:14670 and in Eglom et al. (1993) Nature 365:566. One advantage of PNA oligomers is their capability to bind to complementary DNA essentially independently from the ionic strength of the medium due to the neutral backbone of the DNA. In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet a further embodiment, the antisense oligonucleotide is an -anomeric oligonucleotide. An -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-0-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g., by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

While antisense nucleotides complementary to the coding region of an mRNA sequence can be used, those complementary to the transcribed untranslated region and to the region comprising the initiating methionine are most preferred.

The antisense molecules can be delivered to cells that express *hedgehog* signaling genes in vivo. A number of methods have been developed for delivering antisense DNA or RNA to cells; e.g., antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (e.g., antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systematically.

However, it may be difficult to achieve intracellular concentrations of the antisense sufficient to suppress translation on endogenous mRNAs in certain instances. Therefore a

preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous *hedgehog* signaling transcripts and thereby prevent translation. For example, a vector can be introduced in vivo such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al, 1982, Nature 296:39-42), etc. Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct that can be introduced directly into the tissue site. Alternatively, viral vectors can be used which selectively infect the desired tissue, in which case administration may be accomplished by another route (e.g., systematically).

Ribozyme molecules designed to catalytically cleave *hedgehog* signaling mRNA transcripts can also be used to prevent translation of mRNA (See, e.g., PCT International Publication WO90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225 and U.S. Patent No. 5,093,246). While ribozymes that cleave mRNA at site-specific recognition sequences can be used to destroy particular mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, 1988, Nature, 334:585-591.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in Tetrahymena thermophila (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug, et al., 1984, Science, 224:574-578; Zaug and Cech, 1986, Science, 231:470-475; Zaug, et al., 1986, Nature, 324:429-433; published International patent application No. WO88/04300 by University Patents Inc.; Been and Cech, 1986, Cell, 47:207-216). The Cech-type ribozymes have an eight base pair active site that hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes that target eight base-pair active site sequences.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (e.g., for improved stability, targeting, etc.) and should be delivered to cells that express *hedgehog* signaling genes in vivo. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy targeted messages and inhibit translation. Because ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Alternatively, endogenous *hedgehog* signaling gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the gene (i.e., the promoter and/or enhancers) to form triple helical structures that prevent transcription of the gene in target cells in the body. (See generally, Helene, C. 1991, Anticancer Drug Des., 6(6):569-84; Helene, C., et al., 1992, Ann. N.Y. Acad. Sci., 660:27-36; and Maher, L.J., 1992, Bioassays 14(12):807-15).

Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription are preferably single stranded and composed of deoxyribonucleotides. The base composition of these oligonucleotides should promote triple helix formation via Hoogsteen base pairing rules, which generally require sizable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules

may be chosen that are purine- rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in CGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so-called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizable stretch of either purines or pyrimidines to be present on one strand of a duplex.

Antisense RNA and DNA, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Moreover, various well-known modifications to nucleic acid molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

In general, it is anticipated that methods decreasing the presence or translation of *hedgehog, smoothened* or *gli-1* mRNA will act as *hedgehog* antagonists, while methods that decrease the production of *patched* will have an agonist effect.

In certain embodiments, the subject antagonists can be chosen on the basis of their selectively for the *hedgehog* pathway. This selectivity can be for the *hedgehog* pathway versus

other pathways, or for selectivity between particular *hedgehog* pathways, e.g., e.g., *ptc*-1, *ptc*-2, etc.

In certain preferred embodiments, the subject inhibitors inhibit *hedgehog*-mediated signal transduction with an ED_{50} of 1 mM or less, more preferably of 1 μM or less, and even more preferably of 1 nM or less.

In particular embodiments, the small molecule is chosen for use because it is more selective for one *patched* isoform over the next, e.g., 10 fold, and more preferably at least 100 or even 1000 fold more selective for one *patched* pathway (*ptc*-1, *ptc*-2) over another.

In certain embodiments, a compound which is an antagonist of the *hedgehog* pathway is chosen to selectively antagonize *hedgehog* activity over protein kinases other than PKA, such as PKC, e.g., the compound modulates the activity of the *hedgehog* pathway at least an order of magnitude more strongly than it modulates the activity of another protein kinase, preferably at least two orders of magnitude more strongly, even more preferably at least three orders of magnitude more strongly. Thus, for example, a preferred inhibitor of the *hedgehog* pathway may inhibit *hedgehog* activity with a K_i at least an order of magnitude lower than its K_i for inhibition of PKC, preferably at least two orders of magnitude lower, even more preferably at least three orders of magnitude lower. In certain embodiments, the K_i for PKA inhibition is less than 10 nM, preferably less than 1 nM, even more preferably less than 0.1 nM.

IV. Exemplary Applications of Method and Compositions

Another aspect of the present invention relates to methods of modulating a differentiated state, survival, and/or proliferation of a cell.

For example, it is contemplated that the subject method could be used to inhibit angiogenesis. *Hedgehog* is known to stimulate angiogenesis. Matrigel plugs impregnated with *hedgehog* protein and inserted into mice evince substantial neovascularization, whereas Matrigel plugs not carrying *hedgehog* show comparatively little vascularization. *Hedgehog* protein is also capable of increasing vascularization of the normally avascular mouse cornea. The *ptc-1* gene is expressed in normal vascular tissues, including the endothelial cells of the aorta, vascular smooth muscle cells, adventitial fibroblasts of the aorta, the coronary vasculature and cardiomyocytes of

the atria and ventricles. These tissues are also sensitive to *hedgehog* protein. Treatment with exogenous *hedgehog* causes upregulation of *ptc-1* expression. In addition, *hedgehog* proteins stimulate proliferation of vascular smooth muscle cells in vivo. *Hedgehog* proteins also cause fibroblasts to increase expression of angiogenic growth factors such as VEGF, bFGF, Ang-1 and Ang-2. Lastly, *hedgehog* proteins are known to stimulate recovery from ischemic injury and stimulate formation of collateral vessels.

Given that *hedgehog* promotes angiogenesis, *hedgehog* antagonists are expected to act as angiogenesis inhibitors, particularly in situations where some level of *hedgehog* signaling is necessary for angiogenesis.

Angiogenesis is fundamental to many disorders. Persistent, unregulated angiogenesis occurs in a range of disease states, tumor metastases and abnormal growths by endothelial cells. The vasculature created as a result of angiogenic processes supports the pathological damage seen in these conditions. The diverse pathological states created due to unregulated angiogenesis have been grouped together as angiogenic dependent or angiogenic associated diseases. Therapies directed at control of the angiogenic processes could lead to the abrogation or mitigation of these diseases.

Diseases caused by, supported by or associated with angiogenesis include ocular neovascular disease, age-related macular degeneration, diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, epidemnic keratoconjunctivitis, Vitamin A deficiency, contact lens overwear, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, sjogrens, acne rosacea, phylectenulosis, syphilis, Mycobacteria infections, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, Herpes simplex infections, Herpes zoster infections, protozoan infections, Kaposi sarcoma, Mooren ulcer, Terrien's marginal degeneration, mariginal keratolysis, rheumatoid arthritis, systemic lupus, polyarteritis, trauma, Wegeners sarcoidosis, Scleritis, Steven's Johnson disease, periphigoid radial keratotomy, corneal graph rejection, rheumatoid arthritis, osteoarthritis chronic inflammation (e.g., ulcerative colitis or Crohn's disease), hemangioma, Osler-Weber-Rendu disease, and hereditary hemorrhagic telangiectasia.

In addition, angiogenesis plays a critical role in cancer. A tumor cannot expand without a blood supply to provide nutrients and remove cellular wastes. Tumors in which angiogenesis is

important include solid tumors such as rhabdomyosarcomas, retinoblastoma, Ewing sarcoma, neuroblastoma, and osteosarcoma, and benign tumors such as acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas. Angiogenic factors have been found associated with several solid tumors . Prevention of angiogenesis could halt the growth of these tumors and the resultant damage to the animal due to the presence of the tumor. Angiogenesis is also associated with blood-born tumors such as leukemias, any of various acute or chronic neoplastic diseases of the bone marrow in which unrestrained proliferation of white blood cells occurs, usually accompanied by anemia, impaired blood clotting, and enlargement of the lymph nodes, liver, and spleen. It is believed that angiogenesis plays a role in the abnormalities in the bone marrow that give rise to leukemia-like tumors.

In addition to tumor growth, angiogenesis is important in metastasis. Initially, angiogenesis is important is in the vascularization of the tumor which allows cancerous cells to enter the blood stream and to circulate throughout the body. After the tumor cells have left the primary site, and have settled into the secondary, metastasis site, angiogenesis must occur before the new tumor can grow and expand. Therefore, prevention of angiogenesis could lead to the prevention of metastasis of tumors and possibly contain the neoplastic growth at the primary site.

Angiogenesis is also involved in normal physiological processes such as reproduction and wound healing. Angiogenesis is an important step in ovulation and also in implantation of the blastula after fertilization. Prevention of angiogenesis could be used to induce amenorrhea, to block ovulation or to prevent implantation by the blastula.

It is anticipated that the invention will be useful for the treatment and/or prevention of respiratory distress syndrome or other disorders resulting from inappropriate lung surface tension. Respiratory distress syndrome results from insufficient surfactant in the alveolae of the lungs. The lungs of vertebrates contain surfactant, a complex mixture of lipids and protein that causes surface tension to rise during lung inflation and decrease during lung deflation. During lung deflation, surfactant decreases such that there are no surface forces that would otherwise promote alveolar collapse. Aerated alveoli that have not collapsed during expiration permit continuous oxygen and carbon dioxide transport between blood and alveolar gas and require much less force to inflate during the subsequent inspiration. During inflation, lung surfactant increases surface tension as the alveolar surface area increases. A rising surface tension in

expanding alveoli opposes over-inflation in those airspaces and tends to divert inspired air to less well-aerated alveoli, thereby facilitating even lung aeration.

Respiratory distress syndrome is particularly prevalent among premature infants. Lung surfactant is normally synthesized at a very low rate until the last six weeks of fetal life. Human infants born more than six weeks before the normal term of a pregnancy have a high risk of being born with inadequate amounts of lung surfactant and inadequate rates of surfactant synthesis. The more prematurely an infant is born, the more severe the surfactant deficiency is likely to be. Severe surfactant deficiency can lead to respiratory failure within a few minutes or hours of birth. The surfactant deficiency produces progressive collapse of alveoli (atelectasis) because of the decreasing ability of the lung to expand despite maximum inspiratory effort. As a result, inadequate amounts of oxygen reach the infant's blood. RDS can occur in adults as well, typically as a consequence of failure in surfactant biosynthesis.

Lung tissue of premature infants shows high activity of the *hedgehog* signaling pathway. Inhibition of this pathway using *hedgehog* antagonists increases the formation of *lamellated* bodies and increases the expression of genes involved in surfactant biosynthesis. Lamellar bodies are subcellular structures associated with surfactant biosynthesis. For these reasons, treatment of premature infants with a *hedgehog* antagonist should stimulate surfactant biosynthesis and ameliorate RDS. In cases where adult RDS is associated with *hedgehog* pathway activation, treatment with *hedgehog* antagonists should also be effective.

It is further contemplated that the use of *hedgehog* antagonists may be specifically targeted to disorders where the affected tissue and/or cells evince high *hedgehog* pathway activation. Expression of *gli* genes is activated by the *hedgehog* signaling pathway, including *gli-1*, *gli-2* and *gli-3*. *gli-1* expression is most consistently correlated with *hedgehog* signaling activity across a wide range of tissues and disorders, while *gli-3* is somewhat less so. The *gli* genes encode transcription factors that activate expression of many genes needed to elicit the full effects of *hedgehog* signaling. However, the *Gli*-3 transcription factor can also act as a repressor of *hedgehog* effector genes, and therefore, expression of *gli-3* can cause a decreased effect of the *hedgehog* signaling pathway. Whether *Gli*-3 acts as a transcriptional activator or repressor depends on post-translational events, and therefore it is expected that methods for detecting the activating form (versus the repressing form) of *Gli*-3 protein would also be a reliable measure of *hedgehog* pathway activation. *gli-2* gene expression is expected to provide a reliable marker for

hedgehog pathway activation. The *gli-1* gene is strongly expressed in a wide array of cancers, hyperplasias and immature lungs, and serves as a marker for the relative activation of the *hedgehog* pathway. In addition, tissues, such as immature lung, that have high *gli* gene expression are strongly affected by *hedgehog* inhibitors. Accordingly, it is contemplated that the detection of *gli* gene expression may be used as a powerful predictive tool to identify tissues and disorders that will particularly benefit from treatment with a *hedgehog* antagonist.

In preferred embodiments, *gli-1* expression levels are detected, either by direct detection of the transcript or by detection of protein levels or activity. Transcripts may be detected using any of a wide range of techniques that depend primarily on hybridization of probes to the *gli*-1 transcripts or to cDNAs synthesized therefrom. Well known techniques include Northern blotting, reverse-transcriptase PCR and microarray analysis of transcript levels. Methods for detecting *Gli* protein levels include Western blotting, immunoprecipitation, two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE) (preferably compared against a standard wherein the position of the *Gli* proteins has been determined), and mass spectroscopy. Mass spectroscopy may be coupled with a series of purification steps to allow high-throughput identification of many different protein levels in a particular sample. Mass spectroscopy and 2D SDS-PAGE can also be used to identify post-transcriptional modifications to proteins including proteolytic events, ubiquitination, phosphorylation, lipid modification etc. *Gli* activity may also be assessed by analyzing binding to substrate DNA or in vitro transcriptional activation of target promoters. Gel shift assays, DNA footprinting assays and DNA-protein crosslinking assays are all methods that may be used to assess the presence of a protein capable of binding to *Gli* binding sites on DNA. (*J Mol Med* 1999 Jun; 77(6):459-68; *Cell* 2000 Feb 18;100(4):423-34; *Development* 2000;127(19):4293-4301)

In preferred embodiments, *gli* transcript levels are measured and diseased or disordered tissues showing abnormally high *gli* levels are treated with a *hedgehog* antagonist. Premature lung tissue, lung cancers (e.g., adenocarcinomas, broncho-alveolar adenocarcinomas, small cell carcinomas), breast cancers (e.g., inferior ductal carcinomas, inferior lobular carcinomas, tubular carcinomas), prostate cancers (e.g., adenocarcinomas), and benign prostatic hyperplasias all show strongly elevated *gli-1* expression levels in certain cases. Accordingly, *gli*-1 expression levels are a powerful diagnostic device to determine which of these tissues should be treated with a *hedgehog* antagonist. In addition, there is substantial correlative evidence that cancers of

urothelial cells (e.g., bladder cancer, other urogenital cancers) will also have elevated *gli-1* levels in certain cases. For example, it is known that loss of heterozygosity on chromosome 9q22 is common in bladder cancers. The *ptc-1* gene is located at this position and *ptc-1* loss of function is probably a partial cause of hyperproliferation, as in many other cancer types. Accordingly, such cancers would also show high *gli* expression and would be particularly amenable to treatment with a *hedgehog* antagonist.

Expression of *ptc-1* and *ptc-2* is also activated by the *hedgehog* signaling pathway, but these genes are inferior to the *gli* genes as markers of *hedgehog* pathway activation. In certain tissues only one of *ptc-1* or *ptc-2* is expressed although the *hedgehog* pathway is highly active. For example, in testicular development, *indian hedgehog* plays an important role and the *hedgehog* pathway is activated, but only *ptc-2* is expressed. Accordingly, these genes are individually unreliable as markers for *hedgehog* pathway activation, although simultaneous measurement of both genes is contemplated as a useful indicator for tissues to be treated with a *hedgehog* antagonist.

It is anticipated that any degree of *gli* overexpression may be useful in determining that a hedgehog antagonist will be an effective therapeutic. In preferred embodiments, *gli* should be expressed at a level at least twice as high as normal. In particularly preferred embodiments, expression is four, six, eight or ten times as high as normal.

For instance, it is contemplated by the invention that, in light of the findings of an apparently broad involvement of *hedgehog*, *ptc*, and *smoothened* in the formation of ordered spatial arrangements of differentiated tissues in vertebrates, the subject method could be used as part of a process for generating and/or maintaining an array of different vertebrate tissue both *in vitro* and *in vivo*. The *hedgehog* antagonist, whether inductive or anti-inductive with respect to proliferation or differentiation of a given tissue, can be, as appropriate, any of the preparations described above.

For example, the present method is applicable to cell culture techniques wherein it is desirable to reduce the level of *hedgehog* signaling. *In vitro* neuronal culture systems have proved to be fundamental and indispensable tools for the study of neural development, as well as the identification of neurotrophic factors such as nerve growth factor (NGF), ciliary trophic factors (CNTF), and brain derived neurotrophic factor (BDNF). One use of the present method may be in cultures of neuronal stem cells, such as in the use of such cultures for the generation of

new neurons and glia. In such embodiments of the subject method, the cultured cells can be contacted with a *hedgehog* antagonist of the present invention in order to alter the rate of proliferation of neuronal stem cells in the culture and/or alter the rate of differentiation, or to maintain the integrity of a culture of certain terminally differentiated neuronal cells. In an exemplary embodiment, the subject method can be used to culture, for example, sensory neurons or, alternatively, motor neurons. Such neuronal cultures can be used as convenient assay systems as well as sources of implantable cells for therapeutic treatments.

To further illustrate other uses of the subject *hedgehog* antagonists, it is noted that intracerebral grafting has emerged as an additional approach to central nervous system therapies. For example, one approach to repairing damaged brain tissues involves the transplantation of cells from fetal or neonatal animals into the adult brain (Dunnett et al. (1987) *J Exp Biol* 123:265-289; and Freund et al. (1985) *J Neurosci* 5:603-616). Fetal neurons from a variety of brain regions can be successfully incorporated into the adult brain, and such grafts can alleviate behavioral defects. For example, movement disorder induced by lesions of dopaminergic projections to the basal ganglia can be prevented by grafts of embryonic dopaminergic neurons. Complex cognitive functions that are impaired after lesions of the neocortex can also be partially restored by grafts of embryonic cortical cells. The subject method can be used to regulate the growth state in the culture, or where fetal tissue is used, especially neuronal stem cells, can be used to regulate the rate of differentiation of the stem cells.

Stem cells useful in the present invention are generally known. For example, several neural crest cells have been identified, some of which are multipotent and likely represent uncommitted neural crest cells, and others of which can generate only one type of cell, such as sensory neurons, and likely represent committed progenitor cells. The role of *hedgehog* antagonists employed in the present method to culture such stem cells can be to regulate differentiation of the uncommitted progenitor, or to regulate further restriction of the developmental fate of a committed progenitor cell towards becoming a terminally differentiated neuronal cell. For example, the present method can be used *in vitro* to regulate the differentiation of neural crest cells into glial cells, schwann cells, chromaffin cells, cholinergic sympathetic or parasympathetic neurons, as well as peptidergic and serotonergic neurons. The *hedgehog*

antagonists can be used alone, or can be used in combination with other neurotrophic factors that act to more particularly enhance a particular differentiation fate of the neuronal progenitor cell.

In addition to the implantation of cells cultured in the presence of the subject *hedgehog* antagonists, yet another aspect of the present invention concerns the therapeutic application of a *hedgehog* antagonist to regulate the growth state of neurons and other neuronal cells in both the central nervous system and the peripheral nervous system. The ability of *ptc*, *hedgehog*, and *smoothened* to regulate neuronal differentiation during development of the nervous system and also presumably in the adult state indicates that, in certain instances, the subject *hedgehog* antagonists can be expected to facilitate control of adult neurons with regard to maintenance, functional performance, and aging of normal cells; repair and regeneration processes in chemically or mechanically lesioned cells; and treatment of degeneration in certain pathological conditions. In light of this understanding, the present invention specifically contemplates applications of the subject method to the treatment protocol of (prevention and/or reduction of the severity of) neurological conditions deriving from: (i) acute, subacute, or chronic injury to the nervous system, including traumatic injury, chemical injury, vascular injury and deficits (such as the ischemia resulting from stroke), together with infectious/inflammatory and tumor-induced injury; (ii) aging of the nervous system including Alzheimer's disease; (iii) chronic neurodegenerative diseases of the nervous system, including Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis and the like, as well as spinocerebellar degenerations; and (iv) chronic immunological diseases of the nervous system or affecting the nervous system, including multiple sclerosis.

As appropriate, the subject method can also be used in generating nerve prostheses for the repair of central and peripheral nerve damage. In particular, where a crushed or severed axon is intubulated by use of a prosthetic device, *hedgehog* antagonists can be added to the prosthetic device to regulate the rate of growth and regeneration of the dendritic processes. Exemplary nerve guidance channels are described in U.S. patents 5,092,871 and 4,955,892.

In another embodiment, the subject method can be used in the treatment of neoplastic or hyperplastic transformations such as may occur in the central nervous system. For instance, the *hedgehog* antagonists can be utilized to cause such transformed cells to become either post-mitotic or apoptotic. The present method may, therefore, be used as part of a treatment for, e.g.,

malignant gliomas, meningiomas, medulloblastomas, neuroectodermal tumors, and ependymomas.

In a preferred embodiment, the subject method can be used as part of a treatment regimen for malignant medulloblastoma and other primary CNS malignant neuroectodermal tumors.

In certain embodiments, the subject method is used as part of treatment program for medulloblastoma. Medulloblastoma, a primary brain tumor, is the most common brain tumor in children. A medulloblastoma is a primitive neuroectodermal tumor arising in the posterior fossa. They account for approximately 25% of all pediatric brain tumors (Miller). Histologically, they are small round cell tumors commonly arranged in true rosettes, but may display some differentiation to astrocytes, ependymal cells or neurons (Rorke; Kleihues). PNET's may arise in other areas of the brain including the pineal gland (pineoblastoma) and cerebrum. Those arising in the supratentorial region generally fare worse than their PF counterparts.

Medulloblastoma/PNET's are known to recur anywhere in the CNS after resection, and can even metastasize to bone. Pretreatment evaluation should therefore include an examination of the spinal cord to exclude the possibility of "dropped metastases". Gadolinium-enhanced MRI has largely replaced myelography for this purpose, and CSF cytology is obtained postoperatively as a routine procedure.

In other embodiments, the subject method is used as part of treatment program for ependymomas. Ependymomas account for approximately 10% of the pediatric brain tumors in children. Grossly, they are tumors that arise from the ependymal lining of the ventricles and microscopically form rosettes, canals, and perivascular rosettes. In the CHOP series of 51 children reported with ependymomas, ¾ were histologically benign. Approximately 2/3 arose from the region of the 4th ventricle. One third presented in the supratentorial region. Age at presentation peaks between birth and 4 years, as demonstrated by SEER data as well as data from CHOP. The median age is about 5 years. Because so many children with this disease are babies, they often require multimodal therapy.

Yet another aspect of the present invention concerns the observation in the art that *ptc*, *hedgehog*, and/or *smoothened* are involved in morphogenic signals involved in other vertebrate organogenic pathways in addition to neuronal differentiation as described above, having apparent

roles in other endodermal patterning, as well as both mesodermal and endodermal differentiation processes. Thus, it is contemplated by the invention that compositions comprising *hedgehog* antagonists can also be utilized for both cell culture and therapeutic methods involving generation and maintenance of non-neuronal tissue.

In one embodiment, the present invention makes use of the discovery that *ptc*, *hedgehog*, and *smoothened* are apparently involved in controlling the development of stem cells responsible for formation of the digestive tract, liver, lungs, and other organs which derive from the primitive gut. *Shh* serves as an inductive signal from the endoderm to the mesoderm, which is critical to gut morphogenesis. Therefore, for example, *hedgehog* antagonists of the instant method can be employed for regulating the development and maintenance of an artificial liver that can have multiple metabolic functions of a normal liver. In an exemplary embodiment, the subject method can be used to regulate the proliferation and differentiation of digestive tube stem cells to form hepatocyte cultures which can be used to populate extracellular matrices, or which can be encapsulated in biocompatible polymers, to form both implantable and extracorporeal artificial livers.

In another embodiment, therapeutic compositions of *hedgehog* antagonists can be utilized in conjunction with transplantation of such artificial livers, as well as embryonic liver structures, to regulate uptake of intraperitoneal implantation, vascularization, and *in vivo* differentiation and maintenance of the engrafted liver tissue.

In yet another embodiment, the subject method can be employed therapeutically to regulate such organs after physical, chemical or pathological insult. For instance, therapeutic compositions comprising *hedgehog* antagonists can be utilized in liver repair subsequent to a partial hepatectomy.

The generation of the pancreas and small intestine from the embryonic gut depends on intercellular signalling between the endodermal and mesodermal cells of the gut. In particular, the differentiation of intestinal mesoderm into smooth muscle has been suggested to depend on signals from adjacent endodermal cells. One candidate mediator of endodermally derived signals in the embryonic hindgut is Sonic *hedgehog*. See, for example, Apelqvist et al. (1997) Curr Biol 7:801-4. The Shh gene is expressed throughout the embryonic gut endoderm with the exception of the pancreatic bud endoderm, which instead expresses high levels of the homeodomain protein

Ipfl/Pdx1 (insulin promoter factor 1/pancreatic and duodenal homeobox 1), an essential regulator of early pancreatic development. Apelqvist et al., supra, have examined whether the differential expression of Shh in the embryonic gut tube controls the differentiation of the surrounding mesoderm into specialised mesoderm derivatives of the small intestine and pancreas. To test this, they used the promoter of the Ipfl/Pdx1 gene to selectively express Shh in the developing pancreatic epithelium. In Ipfl/Pdx1-Shh transgenic mice, the pancreatic mesoderm developed into smooth muscle and interstitial cells of Cajal, characteristic of the intestine, rather than into pancreatic mesenchyme and spleen. Also, pancreatic explants exposed to Shh underwent a similar program of intestinal differentiation. These results provide evidence that the differential expression of endodermally derived Shh controls the fate of adjacent mesoderm at different regions of the gut tube.

In the context of the present invention, it is contemplated therefore that the subject *hedgehog* antagonists can be used to control or regulate the proliferation and/or differentiation of pancreatic tissue both *in vivo* and *in vitro*.

In another embodiment, hedgehog antagonists are used to generate endodermal tissue from non-endodermal stem cells including mesenchymal stem cells and stem cells derived from mesodermal tissues. Exemplary mesodermal tissues from which stem cells may be isolated include skeletal muscle, cardiac muscle, kidney, bone, cartilage, and fat.

There are a wide variety of pathological cell proliferative and differentiative conditions for which the inhibitors of the present invention may provide therapeutic benefits, with the general strategy being, for example, the correction of aberrant insulin expression, or modulation of differentiation. More generally, however, the present invention relates to a method of inducing and/or maintaining a differentiated state, enhancing survival and/or affecting proliferation of pancreatic cells, by contacting the cells with the subject inhibitors. For instance, it is contemplated by the invention that, in light of the apparent involvement of *ptc*, *hedgehog*, and *smoothened* in the formation of ordered spatial arrangements of pancreatic tissues, the subject method could be used as part of a technique to generate and/or maintain such tissue both *in vitro* and *in vivo*. For instance, modulation of the function of *hedgehog* can be employed in both cell culture and therapeutic methods involving generation and maintenance of β-cells and possibly also for non-pancreatic tissue, such as in controlling the development and maintenance of tissue

from the digestive tract, spleen, lungs, urogenital organs (e.g., bladder), and other organs which derive from the primitive gut.

In an exemplary embodiment, the present method can be used in the treatment of hyperplastic and neoplastic disorders effecting pancreatic tissue, particularly those characterized by aberrant proliferation of pancreatic cells. For instance, pancreatic cancers are marked by abnormal proliferation of pancreatic cells, which can result in alterations of insulin secretory capacity of the pancreas. For instance, certain pancreatic hyperplasias, such as pancreatic carcinomas, can result in hypoinsulinemia due to dysfunction of β-cells or decreased islet cell mass.

Moreover, manipulation of *hedgehog* signaling properties at different points may be useful as part of a strategy for reshaping/repairing pancreatic tissue both *in vivo* and *in vitro*. In one embodiment, the present invention makes use of the apparent involvement of *ptc*, *hedgehog*, and *smoothened* in regulating the development of pancreatic tissue. In general, the subject method can be employed therapeutically to regulate the pancreas after physical, chemical or pathological insult. In yet another embodiment, the subject method can be applied to cell culture techniques, and in particular, may be employed to enhance the initial generation of prosthetic pancreatic tissue devices. Manipulation of proliferation and differentiation of pancreatic tissue, for example, by altering *hedgehog* activity, can provide a means for more carefully controlling the characteristics of a cultured tissue. In an exemplary embodiment, the subject method can be used to augment production of prosthetic devices which require β-islet cells, such as may be used in the encapsulation devices described in, for example, the Aebischer et al. U.S. Patent No. 4,892,538, the Aebischer et al. U.S. Patent No. 5,106,627, the Lim U.S. Patent No. 4,391,909, and the Sefton U.S. Patent No. 4,353,888. Early progenitor cells to the pancreatic islets are multipotential, and apparently coactivate all the islet-specific genes from the time they first appear. As development proceeds, expression of islet-specific hormones, such as insulin, becomes restricted to the pattern of expression characteristic of mature islet cells. The phenotype of mature islet cells, however, is not stable in culture, as reappearance of embryonal traits in mature β-cells can be observed. By utilizing the subject *hedgehog* antagonists, the differentiation path or proliferative index of the cells can be regulated.

Furthermore, manipulation of the differentiative state of pancreatic tissue can be utilized in conjunction with transplantation of artificial pancreas. For instance, manipulation of *hedgehog* function to affect tissue differentiation can be utilized as a means of maintaining graft viability.

Bellusci et al. (1997) Development 124:53 report that *Sonic hedgehog* regulates lung mesenchymal cell proliferation in vivo. Accordingly, the present method can be used to regulate regeneration of lung tissue, e.g., in the treatment of emphysema.

Fujita et al. (1997) *Biochem Biophys Res Commun* 238:658 reported that Sonic *hedgehog* is expressed in human lung squamous carcinoma and adenocarcinoma cells. The expression of Sonic *hedgehog* was also detected in the human lung squamous carcinoma tissues, but not in the normal lung tissue of the same patient. They also observed that Sonic *hedgehog* stimulates the incorporation of BrdU into the carcinoma cells and stimulates their cell growth, while anti-Shh-N inhibited their cell growth. These results suggest that a *ptc*, *hedgehog*, and/or *smoothened* is involved in the cell growth of such transformed lung tissue and therefore indicates that the subject method can be used as part of a treatment of lung carcinoma and adenocarcinomas, and other proliferative disorders involving the lung epithelia.

Many other tumors may, based on evidence such as involvement of the *hedgehog* pathway in these tumors, or detected expression of *hedgehog* or its receptor in these tissues during development, be affected by treatment with the subject compounds. Such tumors include, but are by no means limited to, tumors related to Gorlin's syndrome (e.g., medulloblastoma, meningioma, etc.), tumors evidenced in *ptc* knock-out mice (e.g., hemangioma, rhabdomyosarcoma, etc.), tumors resulting from *gli-1* amplification (e.g., glioblastoma, sarcoma, etc.), tumors connected with TRC8, a *ptc* homolog (e.g., renal carcinoma, thyroid carcinoma, etc.), *Ext-1*-related tumors (e.g., bone cancer, etc.), Shh-induced tumors (e.g., lung cancer, chondrosarcomas, etc.), and other tumors (e.g., breast cancer, urogenital cancer (e.g., kidney, bladder, ureter, prostate, etc.), adrenal cancer, gastrointestinal cancer (e.g., stomach, intestine, etc.), etc.).

In still another embodiment of the present invention, compositions comprising *hedgehog* antagonists can be used in the *in vitro* generation of skeletal tissue, such as from skeletogenic stem cells, as well as the *in vivo* treatment of skeletal tissue deficiencies. The present invention particularly contemplates the use of *hedgehog* antagonists to regulate the rate of chondrogenesis

and/or osteogenesis. By "skeletal tissue deficiency", it is meant a deficiency in bone or other skeletal connective tissue at any site where it is desired to restore the bone or connective tissue, no matter how the deficiency originated, e.g., whether as a result of surgical intervention, removal of tumor, ulceration, implant, fracture, or other traumatic or degenerative conditions.

For instance, the method of the present invention can be used as part of a regimen for restoring cartilage function to a connective tissue. Such methods are useful in, for example, the repair of defects or lesions in cartilage tissue which is the result of degenerative wear such as that which results in arthritis, as well as other mechanical derangements which may be caused by trauma to the tissue, such as a displacement of torn meniscus tissue, meniscectomy, a laxation of a joint by a torn ligament, malignment of joints, bone fracture, or by hereditary disease. The present reparative method is also useful for remodeling cartilage matrix, such as in plastic or reconstructive surgery, as well as periodontal surgery. The present method may also be applied to improving a previous reparative procedure, for example, following surgical repair of a meniscus, ligament, or cartilage. Furthermore, it may prevent the onset or exacerbation of degenerative disease if applied early enough after trauma.

In one embodiment of the present invention, the subject method comprises treating the afflicted connective tissue with a therapeutically sufficient amount of a *hedgehog* antagonist, particularly an antagonist selective for *India hedgehog* signal transduction, to regulate a cartilage repair response in the connective tissue by managing the rate of differentiation and/or proliferation of chondrocytes embedded in the tissue. Such connective tissues as articular cartilage, interarticular cartilage (menisci), costal cartilage (connecting the true ribs and the sternum), ligaments, and tendons are particularly amenable to treatment in reconstructive and/or regenerative therapies using the subject method. As used herein, regenerative therapies include treatment of degenerative states which have progressed to the point of which impairment of the tissue is obviously manifest, as well as preventive treatments of tissue where degeneration is in its earliest stages or imminent.

In an illustrative embodiment, the subject method can be used as part of a therapeutic intervention in the treatment of cartilage of a diarthroidal joint, such as a knee, an ankle, an elbow, a hip, a wrist, a knuckle of either a finger or toe, or a tempomandibular joint. The treatment can be directed to the meniscus of the joint, to the articular cartilage of the joint, or

both. To further illustrate, the subject method can be used to treat a degenerative disorder of a knee, such as which might be the result of traumatic injury (e.g., a sports injury or excessive wear) or osteoarthritis. The subject antagonists may be administered as an injection into the joint with, for instance, an arthroscopic needle. In some instances, the injected agent can be in the form of a hydrogel or other slow release vehicle described above in order to permit a more extended and regular contact of the agent with the treated tissue.

The present invention further contemplates the use of the subject method in the field of cartilage transplantation and prosthetic device therapies. However, problems arise, for instance, because the characteristics of cartilage and fibrocartilage varies between different tissue: such as between articular, meniscal cartilage, ligaments, and tendons, between the two ends of the same ligament or tendon, and between the superficial and deep parts of the tissue. The zonal arrangement of these tissues may reflect a gradual change in mechanical properties, and failure occurs when implanted tissue, which has not differentiated under those conditions, lacks the ability to appropriately respond. For instance, when meniscal cartilage is used to repair anterior cruciate ligaments, the tissue undergoes a metaplasia to pure fibrous tissue. By regulating the rate of chondrogenesis, the subject method can be used to particularly address this problem, by helping to adaptively control the implanted cells in the new environment and effectively resemble hypertrophic chondrocytes of an earlier developmental stage of the tissue.

In similar fashion, the subject method can be applied to enhancing both the generation of prosthetic cartilage devices and to their implantation. The need for improved treatment has motivated research aimed at creating new cartilage that is based on collagen-glycosaminoglycan templates (Stone et al. (1990) *Clin Orthop Relat Red* 252:129), isolated chondrocytes (Grande et al. (1989) *J Orthop Res* 7:208; and Takigawa et al. (1987) *Bone Miner* 2:449), and chondrocytes attached to natural or synthetic polymers (Walitani et al. (1989) *J Bone Jt Surg* 71B:74; Vacanti et al. (1991) *Plast Reconstr Surg* 88:753; von Schroeder et al. (1991) *J Biomed Mater Res* 25:329; Freed et al. (1993) *J Biomed Mater Res* 27:11; and the Vacanti et al. U.S. Patent No. 5,041,138). For example, chondrocytes can be grown in culture on biodegradable, biocompatible highly porous scaffolds formed from polymers such as polyglycolic acid, polylactic acid, agarose gel, or other polymers that degrade over time as function of hydrolysis of the polymer backbone into innocuous monomers. The matrices are designed to allow adequate nutrient and gas

exchange to the cells until engraftment occurs. The cells can be cultured *in vitro* until adequate cell volume and density has developed for the cells to be implanted. One advantage of the matrices is that they can be cast or molded into a desired shape on an individual basis, so that the final product closely resembles the patient's own ear or nose (by way of example), or flexible matrices can be used which allow for manipulation at the time of implantation, as in a joint.

In one embodiment of the subject method, the implants are contacted with a *hedgehog* antagonist during certain stages of the culturing process in order to manage the rate of differentiation of chondrocytes and the formation of hypertrophic chrondrocytes in the culture.

In another embodiment, the implanted device is treated with a *hedgehog* antagonist in order to actively remodel the implanted matrix and to make it more suitable for its intended function. As set out above with respect to tissue transplants, the artificial transplants suffer from the same deficiency of not being derived in a setting which is comparable to the actual mechanical environment in which the matrix is implanted. The ability to regulate the chondrocytes in the matrix by the subject method can allow the implant to acquire characteristics similar to the tissue for which it is intended to replace.

In yet another embodiment, the subject method is used to enhance attachment of prosthetic devices. To illustrate, the subject method can be used in the implantation of a periodontal prosthesis, wherein the treatment of the surrounding connective tissue stimulates formation of periodontal ligament about the prosthesis.

In still further embodiments, the subject method can be employed as part of a regimen for the generation of bone (osteogenesis) at a site in the animal where such skeletal tissue is deficient. India hedge*hog* is particularly associated with the hypertrophic chondrocytes that are ultimately replaced by osteoblasts. For instance, administration of a *hedgehog* antagonists of the present invention can be employed as part of a method for regulating the rate of bone loss in a subject. For example, preparations comprising *hedgehog* antagonists can be employed, for example, to control endochondral ossification in the formation of a "model" for ossification.

In yet another embodiment of the present invention, a *hedgehog* antagonist can be used to regulate spermatogenesis. The *hedgehog* proteins, particularly Dhh, have been shown to be involved in the differentiation and/or proliferation and maintenance of testicular germ cells. Dhh

expression is initiated in Sertoli cell precursors shortly after the activation of Sry (testicular determining gene) and persists in the testis into the adult. Males are viable but infertile, owing to a complete absence of mature sperm. Examination of the developing testis in different genetic backgrounds suggests that Dhh regulates both early and late stages of spermatogenesis. Bitgood et al. (1996) Curr Biol 6:298. In a preferred embodiment, the *hedgehog* antagonist can be used as a contraceptive. In similar fashion, *hedgehog* antagonists of the subject method are potentially useful for modulating normal ovarian function.

The subject method also has wide applicability to the treatment or prophylaxis of disorders afflicting epithelial tissue, as well as in cosmetic uses. In general, the method can be characterized as including a step of administering to an animal an amount of a *hedgehog* antagonist effective to alter the growth state of a treated epithelial tissue. The mode of administration and dosage regimens will vary depending on the epithelial tissue(s) that is to be treated. For example, topical formulations will be preferred where the treated tissue is epidermal tissue, such as dermal or mucosal tissues.

A method that "promotes the healing of a wound" results in the wound healing more quickly as a result of the treatment than a similar wound heals in the absence of the treatment. "Promotion of wound healing" can also mean that the method regulates the proliferation and/or growth of, *inter alia,* keratinocytes, or that the wound heals with less scarring, less wound contraction, less collagen deposition and more superficial surface area. In certain instances, "promotion of wound healing" can also mean that certain methods of wound healing have improved success rates, (e.g., the take rates of skin grafts,) when used together with the method of the present invention.

Despite significant progress in reconstructive surgical techniques, scarring can be an important obstacle in regaining normal function and appearance of healed skin. This is particularly true when pathologic scarring such as keloids or hypertrophic scars of the hands or face causes functional disability or physical deformity. In the severest circumstances, such scarring may precipitate psychosocial distress and a life of economic deprivation. Wound repair includes the stages of hemostasis, inflammation, proliferation, and remodeling. The proliferative stage involves multiplication of fibroblasts and endothelial and epithelial cells. Through the use of the subject method, the rate of proliferation of epithelial cells in and proximal to the wound

can be controlled in order to accelerate closure of the wound and/or minimize the formation of scar tissue.

The present treatment can also be effective as part of a therapeutic regimen for treating oral and paraoral ulcers, e.g., resulting from radiation and/or chemotherapy. Such ulcers commonly develop within days after chemotherapy or radiation therapy. These ulcers usually begin as small, painful irregularly shaped lesions usually covered by a delicate gray necrotic membrane and surrounded by inflammatory tissue. In many instances, lack of treatment results in proliferation of tissue around the periphery of the lesion on an inflammatory basis. For instance, the epithelium bordering the ulcer usually demonstrates proliferative activity, resulting in loss of continuity of surface epithelium. These lesions, because of their size and loss of epithelial integrity, dispose the body to potential secondary infection. Routine ingestion of food and water becomes a very painful event and, if the ulcers proliferate throughout the alimentary canal, diarrhea usually is evident with all its complicating factors. According to the present invention, a treatment for such ulcers that includes application of a *hedgehog* antagonist can reduce the abnormal proliferation and differentiation of the affected epithelium, helping to reduce the severity of subsequent inflammatory events.

The subject method and compositions can also be used to treat wounds resulting from dermatological diseases, such as lesions resulting from autoimmune disorders such as psoriasis. Atopic dermititis refers to skin trauma resulting from allergies associated with an immune response caused by allergens such as pollens, foods, dander, insect venoms and plant toxins.

In other embodiments, antiproliferative preparations of *hedgehog* antagonists can be used to inhibit lens epithelial cell proliferation to prevent post-operative complications of extracapsular cataract extraction. Cataract is an intractable eye disease and various studies on a treatment of cataract have been made. But at present, the treatment of cataract is attained by surgical operations. Cataract surgery has been applied for a long time and various operative methods have been examined. Extracapsular lens extraction has become the method of choice for removing cataracts. The major medical advantages of this technique over intracapsular extraction are lower incidence of aphakic cystoid macular edema and retinal detachment. Extracapsular extraction is also required for implantation of posterior chamber-type intraocular lenses, which are now considered to be the lenses of choice in most cases.

However, a disadvantage of extracapsular cataract extraction is the high incidence of posterior lens capsule opacification, often called after-cataract, which can occur in up to 50% of cases within three years after surgery. After-cataract is caused by proliferation of equatorial and anterior capsule lens epithelial cells that remain after extracapsular lens extraction. These cells proliferate to cause Sommerling rings, and along with fibroblasts, which also deposit and occur on the posterior capsule, cause opacification of the posterior capsule, which interferes with vision. Prevention of after-cataract would be preferable to treatment. To inhibit secondary cataract formation, the subject method provides a means for inhibiting proliferation of the remaining lens epithelial cells. For example, such cells can be induced to remain quiescent by instilling a solution containing a *hedgehog* antagonist preparation into the anterior chamber of the eye after lens removal. Furthermore, the solution can be osmotically balanced to provide minimal effective dosage when instilled into the anterior chamber of the eye, thereby inhibiting subcapsular epithelial growth with some specificity.

The subject method can also be used in the treatment of corneopathies marked by corneal epithelial cell proliferation, as for example in ocular epithelial disorders such as epithelial downgrowth or squamous cell carcinomas of the ocular surface.

Levine et al. (1997) J Neurosci 17:6277 show that *hedgehog* proteins can regulate mitogenesis and photoreceptor differentiation in the vertebrate retina, and Ihh is a candidate factor from the pigmented epithelium to promote retinal progenitor proliferation and photoreceptor differentiation. Likewise, Jensen et al. (1997) Development 124:363 demonstrated that treatment of cultures of perinatal mouse retinal cells with the amino-terminal fragment of Sonic *hedgehog* protein results in an increase in the proportion of cells that incorporate bromodeoxyuridine, in total cell numbers, and in rod photoreceptors, amacrine cells and Muller glial cells, suggesting that Sonic *hedgehog* promotes the proliferation of retinal precursor cells. Thus, the subject method can be used in the treatment of proliferative diseases of retinal cells and regulate photoreceptor differentiation.

Yet another aspect of the present invention relates to the use of the subject method to control hair growth. Hair is basically composed of keratin, a tough and insoluble protein; its chief strength lies in its disulfide bond of cystine. Each individual hair comprises a cylindrical shaft and a root, and is contained in a follicle, a flask-like depression in the skin. The bottom of

the follicle contains a finger-like projection termed the papilla, which consists of connective tissue from which hair grows, and through which blood vessels supply the cells with nourishment. The shaft is the part that extends outwards from the skin surface, whilst the root has been described as the buried part of the hair. The base of the root expands into the hair bulb, which rests upon the papilla. Cells from which the hair is produced grow in the bulb of the follicle; they are extruded in the form of fibers as the cells proliferate in the follicle. Hair "growth" refers to the formation and elongation of the hair fiber by the dividing cells.

As is well known in the art, the common hair cycle is divided into three stages: anagen, catagen and telogen. During the active phase (anagen), the epidermal stem cells of the dermal papilla divide rapidly. Daughter cells move upward and differentiate to form the concentric layers of the hair itself. The transitional stage, catagen, is marked by the cessation of mitosis of the stem cells in the follicle. The resting stage is known as telogen, where the hair is retained within the scalp for several weeks before an emerging new hair developing below it dislodges the telogen-phase shaft from its follicle. From this model it has become clear that the larger the pool of dividing stem cells that differentiate into hair cells, the more hair growth occurs. Accordingly, methods for increasing or reducing hair growth can be carried out by potentiating or inhibiting, respectively, the proliferation of these stem cells.

In certain embodiments, the subject method can be employed as a way of reducing the growth of human hair as opposed to its conventional removal by cutting, shaving, or depilation. For instance, the present method can be used in the treatment of trichosis characterized by abnormally rapid or dense growth of hair, e.g., hypertrichosis. In an exemplary embodiment, *hedgehog* antagonists can be used to manage hirsutism, a disorder marked by abnormal hairiness. The subject method can also provide a process for extending the duration of depilation.

Moreover, because a *hedgehog* antagonist will often be cytostatic to epithelial cells, rather than cytotoxic, such agents can be used to protect hair follicle cells from cytotoxic agents that require progression into S-phase of the cell-cycle for efficacy, e.g., radiation-induced death. Treatment by the subject method can provide protection by causing the hair follicle cells to become quiescent, e.g., by inhibiting the cells from entering S phase, and thereby preventing the follicle cells from undergoing mitotic catastrophe or programmed cell death. For instance, *hedgehog* antagonists can be used for patients undergoing chemo- or radiation-therapies that

ordinarily result in hair loss. By inhibiting cell-cycle progression during such therapies, the subject treatment can protect hair follicle cells from death, which might otherwise result from activation of cell death programs. After the therapy has concluded, the instant method can also be removed with concomitant relief of the inhibition of follicle cell proliferation.

The subject method can also be used in the treatment of folliculitis, such as folliculitis decalvans, folliculitis ulerythematosa reticulata or keloid folliculitis. For example, a cosmetic preparation of a hedge*hog* antagonist can be applied topically in the treatment of pseudofolliculitis, a chronic disorder occurring most often in the submandibular region of the neck and associated with shaving, the characteristic lesions of which are erythematous papules and pustules containing buried hairs.

In another aspect of the invention, the subject method can be used to induce differentiation and/or inhibit proliferation of epithelially derived tissue. Such forms of these molecules can provide a basis for differentiation therapy for the treatment of hyperplastic and/or neoplastic conditions involving epithelial tissue. For example, such preparations can be used for the treatment of cutaneous diseases in which there is abnormal proliferation or growth of cells of the skin.

For instance, the pharmaceutical preparations of the invention are intended for the treatment of hyperplastic epidermal conditions, such as keratosis, as well as for the treatment of neoplastic epidermal conditions such as those characterized by a high proliferation rate for various skin cancers, as for example squamous cell carcinoma. The subject method can also be used in the treatment of autoimmune diseases affecting the skin, in particular, of dermatological diseases involving morbid proliferation and/or keratinization of the epidermis, as for example, caused by psoriasis or atopic dermatosis.

Many common diseases of the skin, such as psoriasis, squamous cell carcinoma, keratoacanthoma and actinic keratosis are characterized by localized abnormal proliferation and growth. For example, in psoriasis, which is characterized by scaly, red, elevated plaques on the skin, the keratinocytes are known to proliferate much more rapidly than normal and to differentiate less completely.

In one embodiment, the preparations of the present invention are suitable for the treatment of dermatological ailments linked to keratinization disorders causing abnormal

proliferation of skin cells, which disorders may be marked by either inflammatory or non-inflammatory components. To illustrate, therapeutic preparations of a *hedgehog* antagonist, e.g., which promotes quiescence or differentiation can be used to treat varying forms of psoriasis, be they cutaneous, mucosal or ungual. Psoriasis, as described above, is typically characterized by epidermal keratinocytes that display marked proliferative activation and differentiation along a "regenerative" pathway. Treatment with an antiproliferative embodiment of the subject method can be used to reverse the pathological epidermal activation and can provide a basis for sustained remission of the disease.

A variety of other keratotic lesions are also candidates for treatment with the subject method. Actinic keratoses, for example, are superficial inflammatory premalignant tumors arising on sun-exposed and irradiated skin. The lesions are erythematous to brown with variable scaling. Current therapies include excisional and cryosurgery. These treatments are painful, however, and often produce cosmetically unacceptable scarring. Accordingly, treatment of keratosis, such as actinic keratosis, can include application, preferably topical, of a *hedgehog* antagonist composition in amounts sufficient to inhibit hyperproliferation of epidermal/epidermoid cells of the lesion.

Acne represents yet another dermatologic ailment which may be treated by the subject method. Acne vulgaris, for instance, is a multifactor disease most commonly occurring in teenagers and young adults, and is characterized by the appearance of inflammatory and noninflammatory lesions on the face and upper trunk. The basic defect which gives rise to acne vulgaris is hypercornification of the duct of a hyperactive sebaceous gland. Hypercornification blocks the normal mobility of skin and follicle microorganisms, and in so doing, stimulates the release of lipases by *Propinobacterium acnes* and *Staphylococcus epidermidis* bacteria and *Pitrosporum ovale*, a yeast. Treatment with an antiproliferative *hedgehog* antagonist, particularly topical preparations, may be useful for preventing the transitional features of the ducts, e.g., hypercornification, which lead to lesion formation. The subject treatment may further include, for example, antibiotics, retinoids and antiandrogens.

The present invention also provides a method for treating various forms of dermatitis. Dermatitis is a descriptive term referring to poorly demarcated lesions that are either pruritic, erythematous, scaly, blistered, weeping, fissured or crusted. These lesions arise from any of a wide variety of causes. The most common types of dermatitis are atopic, contact and diaper

dermatitis. For instance, seborrheic dermatitis is a chronic, usually pruritic, dermatitis with erythema, dry, moist, or greasy scaling, and yellow-crusted patches on various areas, especially the scalp, with exfoliation of an excessive amount of dry scales. The subject method can also be used in the treatment of stasis dermatitis, an often chronic, usually eczematous dermatitis. Actinic dermatitis is dermatitis that due to exposure to actinic radiation such as that from the sun, ultraviolet waves, or x- or gamma-radiation. According to the present invention, the subject method can be used in the treatment and/or prevention of certain symptoms of dermatitis caused by unwanted proliferation of epithelial cells. Such therapies for these various forms of dermatitis can also include topical and systemic corticosteroids, antipruritics, and antibiotics.

Ailments that may be treated by the subject method are disorders specific to non-humans, such as mange.

In still another embodiment, the subject method can be used in the treatment of human cancers, such as tumors of epithelial tissues such as the skin. For example, *hedgehog* antagonists can be employed in the subject method as part of a treatment for human carcinomas, adenocarcinomas, sarcomas and the like.

In another aspect, the present invention provides pharmaceutical preparations comprising *hedgehog* antagonists. The *hedgehog* antagonists for use in the subject method may be conveniently formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the activity of the *hedgehog* antagonist, its use in the pharmaceutical preparation of the invention is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). These vehicles include injectable "deposit formulations".

Pharmaceutical formulations of the present invention can also include veterinary compositions, e.g., pharmaceutical preparations of the *hedgehog* antagonists suitable for veterinary uses, e.g., for the treatment of livestock or domestic animals, e.g., dogs.

Methods of introduction may also be provided by rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested *in vivo* in recent years for the controlled delivery of drugs, including proteinaceous biopharmaceuticals. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of a *hedgehog* antagonist at a particular target site.

The preparations of the present invention may be given orally, parenterally, topically, or rectally. They are, of course, given by forms suitable for each administration route. For example, they are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, controlled release patch, etc. administration by injection, infusion or inhalation; topical by lotion or ointment; and rectal by suppositories. Oral and topical administrations are preferred.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

These compounds may be administered to humans and other animals for therapy by any suitable route of administration, including orally, nasally, as by, for example, a spray, rectally, intravaginally, parenterally, intracisternally and topically, as by powders, ointments or drops, including buccally and sublingually.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms such as described below or by other conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient that is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the particular compound of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular *hedgehog* antagonist employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

In general, a suitable daily dose of a compound of the invention will be that amount of the compound that is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. Generally, intravenous, intracerebroventricular and subcutaneous doses of the compounds of this invention for a patient will range from about 0.0001 to about 100 mg per kilogram of body weight per day.

If desired, the effective daily dose of the active compound may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

The term "treatment" is intended to encompass also prophylaxis, therapy and cure.

The patient receiving this treatment is any animal in need, including primates, in particular humans, and other mammals such as equines, cattle, swine and sheep; and poultry and pets in general.

The compound of the invention can be administered as such or in admixtures with pharmaceutically acceptable and/or sterile carriers and can also be administered in conjunction with other antimicrobial agents such as penicillins, cephalosporins, aminoglycosides and glycopeptides. Conjunctive therapy thus includes sequential, simultaneous and separate administration of the active compound in a way that the therapeutic effects of the first administered one is not entirely disappeared when the subsequent is administered.

V. Pharmacogenomics

The ability to rapidly assess gene expression in patients promises to radically change the means by which a physician selects an appropriate pharmaceutical for treating a particular disease. Gene expression profiles of diseased tissue can be obtained and therapeutic measures can be selected based on the gene expression profile. This methodology is particularly effective when the molecular mechanism of action for a given therapeutic is known. In other words, if an anti-tumor agent acts by inhibiting a particular oncoprotein, it is desirable to know whether a particular cancer expresses that oncogene before attempting to treat the cancer with the anti-tumor agent. As expression profiling becomes faster, cheaper and more reliable, such information may become a routine part of treatment selection, minimizing fruitless treatment protocols and allowing the more rapid application of appropriate therapeutics.

In addition, if a pool of patients suffering from a certain type of disorder can be segregated into subgroups based on gene expression profiles, drugs can be re-tested for their ability to affect these defined subgroups of patients. Thus drugs that appeared useless in the patient group as a whole may now be found to be useful for patient subgroups. This type of screening may allow the resurrection of failed compounds, the identification of new compounds and the identification of new uses for well-known compounds.

The expression of a particular gene can be assessed in many ways. The level of gene transcript or the level of encoded protein may be determined. The presence of a protein may be determined directly, through methods such as antibody binding, mass spectroscopy and two-dimensional gel electrophoresis, or indirectly, by detecting an activity of the protein, be it a biochemical activity or an effect on the levels of another protein or expression of one or more genes.

Methods for measuring levels of gene transcripts are well known in the art and depend for the most part on hybridization of a single stranded probe to the transcript in question (or a cDNA thereof). Such methods include Northern blotting, using a labeled probe, or PCR amplification of the cDNA (also known as RT-PCR). mRNAs and cDNAs may be labeled according to various methods and hybridized to an oligonucleotide array. Such arrays may contain ordered probes corresponding to one or more genes, and in preferred embodiments, the array contains probes corresponding to all the genes in the genome of the organism from which the RNA was obtained.

A number of methodologies are currently used for the measurement of gene expression. The most sensitive of these methodologies utilizes the polymerase chain reaction (PCR) technique, the details of which are provided in U.S. Pat. No. 4,683,195, U.S. Pat. No. 4,683,202, and U.S. Pat. No. 4,965,188, all to Mullis et al., all of which are specifically incorporated herein by reference. The details of PCR technology, thus, are not included herein. Recently, additional technologies for the amplification of nucleic acids have been described, most of which are based upon isothermal amplification strategies as opposed to the temperature cycling required for PCR. These strategies include, for example, Strand Displacement Amplification (SDA)(U.S. Pat. Nos. 5,455,166 and 5,457,027 both to Walker; Walker et al. (1992) PNAS 89:392; each of which is specifically incorporated herein by reference) and Nucleic Acid Sequence-Based Amplification (NASBA)(U.S. Pat. No. 5,130,238 to Malek et al.; European Patent 525882 to Kievits et al.; both specifically incorporated herein by reference). Each of these amplification technologies are similar in that they employ the use of short, deoxyribonucleic acid primers to define the region of amplification, regardless of the enzymes or specific conditions used.

Until recently, RNA amplification required a separate, additional step and the use of non-thermostable reverse transcriptase enzymes to generate a cDNA capable of being amplified by a thermostable DNA polymerase, such as Taq. The discovery of a recombinant thermostable

enzyme (rTth) capable of coupling reverse transcription of the RNA with DNA amplification in a single enzyme:single reaction procedure greatly simplified and enhanced RNA amplification (see, Myers & Gelfand (1991) Biochemistry 30:7661-7666; U.S. Pat. No. 5,407,800 to Gelfand and Myers, both incorporated herein by reference).

In gene expression analysis with microarrays, an array of "probe" oligonucleotides is contacted with a nucleic acid sample of interest, i.e., target, such as polyA mRNA from a particular tissue type. Contact is carried out under hybridization conditions and unbound nucleic acid is then removed. The resultant pattern of hybridized nucleic acid provides information regarding the genetic profile of the sample tested. Gene expression analysis finds use in a variety of applications, including: the identification of novel expression of genes, the correlation of gene expression to a particular phenotype, screening for disease predisposition, identifying the effect of a particular agent on cellular gene expression, such as in toxicity testing; among other applications. Detailed methods for analyzing transcript levels are described in the following patents: U.S. Pat. No. 5,082,830 and WO 97/27317.

Other references of interest include: Schena et al., Science (1995) 467-470; Schena et al., P.N.A.S. U.S.A. (1996) 93: 10614-10616; Pietu et al., Genome Res. (June 1996) 6: 492-503; Zhao et al., Gene (Apr. 24, 1995) 156: 207-213; Soares, Curr. Opin. Biotechnol. (October 1997) 8: 542-546; Raval, J. Pharmacol Toxicol Methods (November 1994) 32: 125-127; Chalifour et al., Anal. Biochem (Feb. 1, 1994) 216: 299-304; Stolz & Tuan, Mol. Biotechnol. (December 19960 6: 225-230; Hong et al., Bioscience Reports (1982) 2: 907; and McGraw, Anal. Biochem. (1984) 143: 298.

VI. Pharmaceutical Compositions

While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition). The *hedgehog* antagonists according to the invention may be formulated for administration in any convenient way for use in human or veterinary medicine. In certain embodiments, the compound included in the pharmaceutical preparation may be active itself, or may be a prodrug, e.g., capable of being converted to an active compound in a physiological setting.

Thus, another aspect of the present invention provides pharmaceutically acceptable compositions comprising a therapeutically effective amount of one or more of the compounds described above, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. As described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; or (4) intravaginally or intrarectally, for example, as a pessary, cream or foam. However, in certain embodiments the subject compounds may be simply dissolved or suspended in sterile water. In certain embodiments, the pharmaceutical preparation is non-pyrogenic, i.e., does not elevate the body temperature of a patient.

The phrase "therapeutically effective amount" as used herein means that amount of a compound, material, or composition comprising a compound of the present invention which is effective for producing some desired therapeutic effect by overcoming a *hedgehog* gain-of-function phenotype in at least a sub-population of cells in an animal and thereby blocking the biological consequences of that pathway in the treated cells, at a reasonable benefit/risk ratio applicable to any medical treatment.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject antagonists from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other

ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

As set out above, certain embodiments of the present *hedgehog* antagonists may contain a basic functional group, such as amino or alkylamino, and are, thus, capable of forming pharmaceutically acceptable salts with pharmaceutically acceptable acids. The term "pharmaceutically acceptable salts" in this respect, refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds of the present invention. These salts can be prepared *in situ* during the final isolation and purification of the compounds of the invention, or by separately reacting a purified compound of the invention in its free base form with a suitable organic or inorganic acid, and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, phosphate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate salts and the like. (See, for example, Berge et al. (1977) "Pharmaceutical Salts", *J. Pharm. Sci.* 66:1-19)

The pharmaceutically acceptable salts of the subject compounds include the conventional nontoxic salts or quaternary ammonium salts of the compounds, e.g., from non-toxic organic or inorganic acids. For example, such conventional nontoxic salts include those derived from inorganic acids such as hydrochloride, hydrobromic, sulfuric, sulfamic, phosphoric, nitric, and the like; and the salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, palmitic, maleic, hydroxymaleic, phenylacetic,

glutamic, benzoic, salicyclic, sulfanilic, 2-acetoxybenzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isothionic, and the like.

In other cases, the compounds of the present invention may contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically acceptable salts with pharmaceutically acceptable bases. The term "pharmaceutically acceptable salts" in these instances refers to the relatively non-toxic, inorganic and organic base addition salts of compounds of the present invention. These salts can likewise be prepared *in situ* during the final isolation and purification of the compounds, or by separately reacting the purified compound in its free acid form with a suitable base, such as the hydroxide, carbonate or bicarbonate of a pharmaceutically acceptable metal cation, with ammonia, or with a pharmaceutically acceptable organic primary, secondary or tertiary amine. Representative alkali or alkaline earth salts include the lithium, sodium, potassium, calcium, magnesium, and aluminum salts and the like. Representative organic amines useful for the formation of base addition salts include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like. (See, for example, Berge et al., *supra*)

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient that

can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound that produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 1 per cent to about ninety-nine percent of active ingredient, preferably from about 5 per cent to about 70 per cent, most preferably from about 10 per cent to about 30 per cent.

Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present invention with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a compound of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations of the invention suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound of the present invention as an active ingredient. A compound of the present invention may also be administered as a bolus, electuary or paste.

In solid dosage forms of the invention for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules,

tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

The tablets, and other solid dosage forms of the pharmaceutical compositions of the present invention, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions that can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions that can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms for oral administration of the compounds of the invention include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl

alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

It is known that sterols, such as cholesterol, will form complexes with cyclodextrins. Thus, in preferred embodiments, where the inhibitor is a steroidal alkaloid, it may be formulated with cyclodextrins, such as α-, β- and γ-cyclodextrin, dimethyl- β cyclodextrin and 2-hydroxypropyl-β-cyclodextrin.

Formulations of the pharmaceutical compositions of the invention for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active *hedgehog* antagonist.

Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

Dosage forms for the topical or transdermal administration of a compound of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants that may be required.

The ointments, pastes, creams and gels may contain, in addition to an active compound of this invention, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to a compound of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Transdermal patches have the added advantage of providing controlled delivery of a compound of the present invention to the body. Such dosage forms can be made by dissolving or dispersing the *hedgehog* antagonists in the proper medium. Absorption enhancers can also be used to increase the flux of the *hedgehog* antagonists across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the compound in a polymer matrix or gel.

Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention.

Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more compounds of the invention in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers that may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents that delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution, which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of the subject compounds in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions that are compatible with body tissue.

When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

The addition of the active compound of the invention to animal feed is preferably accomplished by preparing an appropriate feed premix containing the active compound in an effective amount and incorporating the premix into the complete ration.

Alternatively, an intermediate concentrate or feed supplement containing the active ingredient can be blended into the feed. The way in which such feed premixes and complete rations can be prepared and administered are described in reference books (such as "Applied

Animal Nutrition", W.H. Freedman and CO., San Francisco, U.S.A., 1969 or "Livestock Feeds and Feeding" O and B books, Corvallis, Ore., U.S.A., 1977).

Examples:

The invention now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1: Hedgehog, lung development and surfactant production

Respiratory distress syndrome results from insufficient surfactant in the alveolae of the lungs. The lungs of vertebrates contain surfactant, a complex mixture of lipids and protein that causes surface tension to rise during lung inflation and decrease during lung deflation. During lung deflation, surfactant decreases such that there are no surface forces that would otherwise promote alveolar collapse. Aerated alveoli that have not collapsed during expiration permit continuous oxygen and carbon dioxide transport between blood and alveolar gas and require much less force to inflate during the subsequent inspiration. During inflation, lung surfactant increases surface tension as the alveolar surface area increases. A rising surface tension in expanding alveoli opposes over-inflation in those airspaces and tends to divert inspired air to less well-aerated alveoli, thereby facilitating even lung aeration.

Respiratory distress syndrome is particularly prevalent among premature infants. Lung surfactant is normally synthesized at a very low rate until the last six weeks of fetal life. Human infants born more than six weeks before the normal term of a pregnancy have a high risk of being born with inadequate amounts of lung surfactant and inadequate rates of surfactant synthesis. The more prematurely an infant is born, the more severe the surfactant deficiency is likely to be. Severe surfactant deficiency can lead to respiratory failure within a few minutes or hours of birth. The surfactant deficiency produces progressive collapse of alveoli (atelectasis) because of the decreasing ability of the lung to expand despite maximum inspiratory effort. As a result, inadequate amounts of oxygen reach the infant's blood. RDS can occur in adults as well, typically as a consequence of failure in surfactant biosynthesis.

The role of the *hedgehog* signaling pathway in lung maturation and surfactant production was investigated, with the finding that inhibition of the *hedgehog* signaling pathway stimulated surfactant production.

The expression of a *hedgehog*-regulated gene, *Gli*-1, was assessed in embryonic mouse lung tissue. *Gli*-1 was strongly expressed in the embryonic lung, however this expression decreases during lung maturation (Figure 4). Note that the decline in *hedgehog* signaling towards the end of embryogenesis correlates with the maturation of the distal lung epithelium into respiratory pneumocytes. *Gli*-1, a transcription factor indicative of *hedgehog* signaling, continues to be expressed in the conducting, but not respiratory airways in the adult.

METHODS: Sections of paraformaldehyde-fixed, paraffin-embedded tissue were cleared, re-hydrated, digested with proteinase K, acetylated and hybridized with [33P]-labeled *sonic hedgehog* and *gli*-1 RNA probes over night, respectively. After high stringency post-hybridization washes, slides were dipped in photo-emulsion, incubated for up to three weeks, developed, and imaged using dark field illumination. Dark-field signals were filled in with artificial color (red) and superimposed with bright-field images.

To further correlate the decrease in *gli-1* expression with lung maturation, expression of *gli-1* was compared to expression of the lung maturation marker, surfactant type C (Sp-C) (Figure 5). This analysis demonstrates that as expression of *gli-1* decreases between E13.5-E16.5, the expression of Sp-C increases.

METHODS: E13.5 and E16.5 mouse lung explants were dissected and analyzed by Quantatative Real-Time PCR (Q-RT-PCR). Briefly, total ribonucleic acid (RNA) is isolated from the tissue and subjected to reverse transcription to generate DNA. This DNA is amplified in a polymerase chain reaction using gene-specific primers as well as primers for the ubiquitously expressed housekeeping gene GAPDH. The two primer sets are labeled with different fluorophores, allowing for quantification of both signals in the same reaction tube in a real-time PCR machine (TaqMan). When calculating the expression levels of *gli*-1 and Sp-C, the specific signal is normalized to the GAPDH signal, which serves as a measure of the total DNA used in the reaction.

As *Gli-1* expression is a marker for *hedgehog* signaling, it appears that the *hedgehog* signaling pathway is active in immature lung tissue. Accordingly, it was hypothesized that

inhibition of the *hedgehog* signaling pathway would permit more rapid lung maturation and, particularly, stimulate surfactant production.

Treatment of embryonic mouse lungs with *hedgehog* antagonist compound B downregulates *Gli-1* expression (Figure 6). METHODS: E13.5 embryonic mouse lungs were dissected. Explants were grown exposed to the air-liquid interface in lung explant medium (DMEM based, additives optimized for the culture of mouse lungs) for 67 hrs. They were then processed for quantitative real-time PCR (Q-RT-PCR). Briefly, total ribonucleic acid (RNA) is isolated from the tissue and subjected to reverse transcription to generate DNA. This DNA is amplified in a polymerase chain reaction using gene-specific primers as well as primers for the ubiquitously expressed housekeeping gene GAPDH. The two primer sets are labeled with different fluorophores, allowing for quantification of both signals in the same reaction tube in a real-time PCR machine (TaqMan). When calculating the expression level of *gli*-1, the specific signal is normalized to the GAPDH signal, which serves as a measure of the total DNA used in the reaction.

Compound B treatment increases surfactant type C production in embryonic mouse lungs (Figure 7). Surfactant production is a measure of lung maturity, and the inability to produce surfactant is the primary cause of adult and infant respiratory distress syndrome. The increase in surfactant type C production was assessed by measuring expression of Sp-C, which encodes a protein critical for the production of surfactant.

METHODS: E13.5 old embryonic mouse lungs were dissected. Explants were grown submerged in lung explant medium (DMEM based, additives optimized for the culture of mouse lungs) for 50 hrs. They were then processed for Q-RT-PCR. Briefly, total ribonucleic acid (RNA) is isolated from the tissue and subjected to reverse transcription to generate DNA. This DNA is amplified in a polymerase chain reaction using gene-specific primers as well as primers for the ubiquitously expressed housekeeping gene GAPDH. The two primer sets are labeled with different fluorophores, allowing for quantification of both signals in the same reaction tube in a real-time PCR machine (TaqMan). When calculating the expression level of Sp-C, the specific signal is normalized to the GAPDH signal, which serves as a measure of the total DNA used in the reaction.

Lamellated bodies are subcellular structures found in surfactin-producing lung cells and are thought to be a site of surfactin production. Type II pneumocytes in compound B-treated lungs differentiate prematurely, as evidenced by the presence of surfactant producing lamellated bodies. No such structures could be observed in the vehicle-treated controls (Figure 8). METHODS: E13.5 old embryonic mouse lungs were dissected. Explants were grown exposed to the air-liquid interface in lung explant medium (DMEM based, additives optimized for the culture of mouse lungs) for 67 hrs. They were then processed for transmission electron microscopy and photographed at a magnification of 62,000.

Figures 9 and 10 show similar results as obtained above upon treatment of embryonic lung cultures with Compound B (Figure 9-10). The increase in Sp-C expression observed following Compound B treatment is comparable to that observed when embryonic lung explants are treated with the steroid hormone hydrocortisone. Steroids are known to increase lung maturation and surfactant production in animals, including humans.

The specificity of the effects of *hedgehog* antagonists on lung maturation is demonstrated by examining the effects of agonists of *hedgehog* signaling on lung maturation. Treatment of embryonic lung cultures with either a lipid modified *sonic hedgehog* or with a *hedgehog* agonist compound result in increased expression of *gli-1* and decreased expression of Sp-C (Figure 11).

In summary, these results demonstrate that *hedgehog* inhibitors can stimulate maturation and surfactin production in immature lung tissue. The *hedgehog* signaling pathway is active in immature lung tissues, where surfactins are not produced in substantial levels, while the *hedgehog* pathway is relatively inactive in the adult respiratory airway, where surfactins are produced. Treatment of immature lung tissue with antagonists of the *hedgehog* signaling pathway causes rapid maturation and the increased presence of molecular and cytological markers associated with surfactin production. Opposite results obtained upon the treatment of lung explants with *hedgehog* antagonists and agonists demonstrate the specificity of these results.

<u>*Example 2: Gli-1 expression in human tumors*</u>

***Hedgehog* pathway activation in human tumors**

Hedgehog signaling plays a causative role in the generation of basal cell carcinoma (BCC). *Hedgehog* signaling was analyzed to determine whether this pathway is active in other human tumors, more specifically prostate, lung and breast cancer, as well as benign prostate hyperplasia. *Hedgehog* proteins are known proliferative agents for a variety of cell types. Since *hedgehogs* have a known proliferative effect on a variety of cell types, *hedgehog* antagonists may be valuable therapeutics for cancers in which high level *hedgehog* signaling is present.

The question of *hedgehog* activation in the tumor types was addressed by conducting radioactive in situ hybridization experiments with *gli*-1, a known transcriptional effector gene of *hedgehog* signaling.

Briefly, sections of paraformaldehyde-fixed, paraffin-embedded tissue were cleared, re-hydrated, digested with proteinase K, acetylated and hybridized with [33P]-labeled RNA probes over night. After high stringency post-hybridization washes, slides were dipped in photo-emulsion, incubated for up to three weeks, developed, and imaged using dark field illumination. Dark-field signals were filled in with artificial color (red) and superimposed with bright-field images. *Gli*-1 expression was graded on a scale from "-" to "+" through "++++". *Gli*-1 expression was rated "-" when expression was no higher in hyperproliferative cells than in other non-proliferative cells present in the slide. Ratings of "+" through "++++" were given for increased expression levels, with any cell rated "++" or above considered to have substantially increased *gli*-1 expression. When the signal was not interpretable, a sample is indicated as "ND".

The data for these experiments are summarized in table 1-4 below. In brief, 8 out of 18 breast cancer samples showed substantially increased *gli*-1 expression. 7 out of 11 lung cancer samples, 11 of 19 benign prostatic hypertrophy samples (BPH), and 6 of 15 prostate cancer samples all showed strong *gli*-1 expression.

Table 1: Results of *Gli-1* in situ hybridization in breast cancer tissue

Tissue	Diagnosis	Sample Number	Age/Sex	Signal
Breast	Inf Ductal Carcinoma	1	93F	ND
Breast	Inf Ductal Carcinoma	2	37F	+++
Breast	Inf Ductal Carcinoma	3	54F	+
Breast	Inf Ductal Carcinoma	4	39F	++
Breast	Inf Ductal Carcinoma	5	73F	+++

Breast	Inf Ductal Carcinoma	6	65F	++++
Breast	Inf Ductal Carcinoma	7	58F	ND
Breast	Inf Ductal Carcinoma	8	48F	+
Breast	Inf Ductal Carcinoma	9	27F	++
Breast	Inf Ductal Carcinoma	10	NA	+++
Breast	Inf Ductal Carcinoma	11	34F	+
Breast	Inf Lobular Carcinoma	12	46F	+
Breast	Inf Lobular Carcinoma	13	F	-
Breast	Inf Lobular Carcinoma	14	56F	+
Breast	Inf Lobular Carcinoma	15	70F	-
Breast	Intraductal Carcinoma	16	40F	+++
Breast	Intraductal Carcinoma	17	55F	+++
Breast	Medullary Carcinoma	18	NA	+
Breast	Tubular Carcinoma	19	75F	-
Breast	Tubular Carcinoma	20	60F	-

Table 2: Results of *Gli-1* in situ hybridization in lung cancer tissue

Tissue	**Diagnosis**	**Sample Number**	**Age/Sex**	**Signal**
Lung	Adenocarcinoma	1	54F	+++++
Lung	Adenocarcinoma	2	61M	ND
Lung	Adenocarcinoma	3	61F	++++
Lung	Adenocarcinoma	4	58F	+++
Lung	Adenocarcinoma	5	77M	ND
Lung	Adenocarcinoma	6	65M	++
Lung	Adenocarcinoma	7	73M	ND
Lung	Adenocarcinoma	8	69M	ND
Lung	Adenocarcinoma	9	82M	ND
Lung	Adenocarcinoma	10	NA	-
Lung	Adenocarcinoma	11	F	ND
Lung	Adenocarcinoma	12	56F	+
Lung	Broncho-alveolar adenocar	13	70F	+
Lung	Broncho-alveolar adenocar	14	76F	-
Lung	Small Cell Carcinoma	15	68M	++
Lung	Small Cell Carcinoma	16	61M	ND
Lung	Small Cell Carcinoma	17	70M	+++++
Lung	Small Cell Carcinoma	18	NA	ND
Lung	SCC	19	60F	ND
Lung	SCC	20	63M	+++++

Table 3: Results of *Gli-1* in situ hybridization in benign prostate hyperplasia

Tissue	Diagnosis	Sample Number	Age/Sex	Signal
Prostate	BPH	1	65M	+

Prostate	BPH	2	86M	++++
Prostate	BPH	3	53M	+
Prostate	BPH	4	65M	++++
Prostate	BPH	5	68M	++
Prostate	BPH	6	70M	++
Prostate	BPH	7	54M	-
Prostate	BPH	8	M	++
Prostate	BPH	9	69M	-
Prostate	BPH	10	M	-
Prostate	BPH	11	73M	+++
Prostate	BPH	12	53M	++++
Prostate	BPH	13	84M	-
Prostate	BPH	14	67M	-
Prostate	BPH	15	66M	++
Prostate	BPH	16	69M	++
Prostate	BPH	17	72M	++++
Prostate	BPH	18	M	++
Prostate	BPH	19	60M	-
Prostate	BPH	20	60M	-

Table 4: Results of *Gli-1* in situ hybridization in prostate cancer tissue

Tissue	Diagnosis	Sample Number	Age/Sex	Signal
Prostate	Adenocarcinoma	1	79M	+
Prostate	Adenocarcinoma	2	72M	+
Prostate	BPH next to Adenocarcinoma	3	81M	ND
Prostate	Adenocarcinoma	4	79M	++
Prostate	Adenocarcinoma	5	81M	ND
Prostate	Adenocarcinoma	6	73M	-
Prostate	Adenocarcinoma	7	79M	++
Prostate	Adenocarcinoma	8	M	+++
Prostate	Adenocarcinoma	9	69M	ND
Prostate	Adenocarcinoma	10	53M	+++
Prostate	Adenocarcinoma	11	65M	+
Prostate	Adenocarcinoma	12	60M	++
Prostate	Adenocarcinoma	13	66M	ND
Prostate	Adenocarcinoma	14	66M	+
Prostate	Adenocarcinoma	15	92M	-
Prostate	Adenocarcinoma	16	80M	-
Prostate	Adenocarcinoma	17	78M	ND
Prostate	Adenocarcinoma	18	85M	-
Prostate	Adenocarcinoma	19	78M	-

Prostate	Adenocarcinoma	20	93M	+++

In summary, high level *Gli*-1 expression, i.e., *hedgehog* signaling activation, can be observed in human prostate cancer and benign prostatic hyperplasia, lung cancer and breast cancer (Figures 12-15). *Hedgehog* pathway activation in these tumor types has never before been described. The presence of an exceptionally active *hedgehog* pathway in these proliferating cells strongly suggests a causal link between the *hedgehog* pathway and hyperproliferation in these disorders. It is expected that *hedgehog* antagonists will be effective as antiproliferative agents in these cancer types.

Example 3: Steroidal hedgehog antagonists

Studies were performed to determine the site in the *hedgehog* signaling pathway at which cyclopamine (an alkaloid steroidal *hedgehog* antagonist) operates, and therefore better understand the spectrum of tumors caused by Shh pathway-activating lesions that could potentially be treated with this compound. These studies are presented in greater detail in U.S. patent application Beachy et al. entitled "*Hedgehog* signaling pathways, compositions and uses related thereto" filed October 10, 2000, the contents of which are herein incorporated by reference.

These studies involve the use of mouse embryonic fibroblasts (MEFs) that were generated by trypsin digestion of E8.5 embryos from *patched* (*ptc*) +/- matings. The mouse *ptc* gene was disrupted by homologous recombination in which part of exon 1 and all of exon 2 were replaced with the bacterial *lacZ* gene (Goodrich *et al*, (1997) Science 277:1109). As *Ptc* protein suppresses Shh signaling, a loss of its function activates the Shh signaling pathway. Shh signaling, through a cascade of events, is mediated by the *Gli* transcription factors. One of the target genes of Shh signaling is *ptc*, through *Gli*-binding sites in the *ptc* promoter region, and this serves as a feedback mechanism for down regulation of signaling. Thus, in these *ptc* -/- embryos, the Shh signaling pathway is activated in many tissues, and the *lacZ* gene product β-galactosidase is expressed in all of those tissues as a report of pathway activation.

These MEFs were obtained to determine whether cyclopamine acts on *Ptc* or another component of the cascade to inhibit Shh signaling. If the target of cyclopamine is *Ptc*, then one

would expect that when the Shh pathway is activated by the loss of *ptc* function, it could no longer be inhibited by cyclopamine. The Shh signaling pathway can be activated in these fibroblasts in cell culture, and that the level of β-galactosidase activity does reflect the degree of pathway activation. The MEF line 23-4 is heterozygous for *ptc-lacZ*, and thus contains one functional *ptc* allele capable of maintaining a repressed state of the pathway, but will express *lacZ* when the pathway is activated by addition of Shh protein.

In contrast, the β-galactosidase activity in MEFs homozygous for *ptc-lacZ*, (cell line 23-1) is markedly elevated, because in these cells the pathway is constitutively activated by the loss of a functional *ptc* allele. When these cells are cultured with cyclopamine, β-galactosidase activity is decreased, indicating that when the Shh signaling pathway is unregulated by *Ptc* repression, it is still sensitive to cyclopamine inhibition. The reduction of β-galactosidase activity appears to result from the specific inhibition of Shh signaling, rather than from cell toxicity because enzymatic activity is normalized to whole protein content of the sample. Also, the reduction of β-galactosidase activity can be obtained with exposure to cyclopamine over a period of time that is shorter than the average cell cycle, and so does not appear to be due solely to an inhibition of cell proliferation.

A final indication that this represents specific inhibition of Shh signaling is that it cannot be achieved with a non-inhibitory, but structurally related compound tomatidine.

Example 4: Lead Compound Discovery/ High-throughput Screening Assay

The methodologies described herein can be used to identify a wide assortment of small molecule *hedgehog* antagonists.

Compounds to be tested are dissolved in DMSO to a concentration of 10 mM, and stored at -20 °C. To activate the *Hedgehog* pathway in the assay cells, an octylated (lipid-modified) form of the N-terminal fragment of the *Sonic Hedgehog* protein (OCT-SHH) is used. This N-terminal SHH fragment is produced bacterially.

Compounds may be tested in the "*Gli*-Luc" assay below, using the cell line 10T(s12), wherein the cells contain a *Hedgehog*-responsive reporter construct utilizing Luciferase as the reporter gene. In this way, *Hedgehog* pathway signaling activity can be measured via the *Gli*-Luc response.

10t1/2(s12) cells are plated in a 96-well micro-titer plate (MTP) at 20,000 cells/well in full medium [DMEM with 10% FBS]. Then plates are placed in the incubator for incubation overnight (O/N), at 37 °C and 5% CO_2. After 24 h, the medium is replaced with Luciferase-assay medium (DMEM with 0.5% FBS). Compounds are thawed and diluted in assay medium at 3:1000 (about 300-fold) resulting in a starting concentration of about 30 μM.

Subsequently, 150 μl of each 30 μM sample is added to the first wells (in triplicate). The MTP samples are then diluted at 3-fold dilutions to a total of seven wells, ultimately resulting in a regiment of seven dilutions in triplicate, for each compound. Next, the protein ligand OCT-SHH is diluted in Luciferase-assay medium and added to each well at a final concentration of 0.3 μg/ml. Plates are then returned to the incubator for further incubation O/N, at 37 °C and 5% CO_2. After about 24 h, plates are removed from the incubator and the medium is aspirated/discarded. Wells are washed once with assay buffer [PBS + 1 mM Mg^{2+} and 1 mM Ca^{2+}]. Then 50 μl of assay buffer is added to each well. The Luciferase assay reagent is prepared as described by the vendor (LucLite kit from Packard), and 50 μl is added to each well. Plates are incubated at room temperature (RT) for about 30 minutes after which the signals are read, again at RT, on a Topcount (Packard).

The discovery of compounds that inhibit Shh-induced *Gli*-transcription exemplifies the utility of the claims in this patent. Activities for these compounds are presented in Table 1 below.

Table 1

Compound	**IC_{50}**	Compound	**IC_{50}**
31	<10 μM	55	<5 μM
32	<5 μM	56	<10 μM
34	<5 μM	57	<10 μM
11	<5 μM	58	<5 μM
36	<5 μM	59	<5 μM
38	<5 μM	60	<5 μM
39	<5 μM	61	<1 μM
40	<10 μM	62	<1 μM
41	<10 μM	63	<10 μM
42	<5 μM	64	<10 μM
43	<10 μM	65	<10 μM

44	<1 μM	66	<10 μM
45	<5 μM	67	<5 μM
46	<0.5 μM	68	<1 μM
47	<5 μM	69	<0.5 μM
48	<0.5 μM	5	<0.1 μM
49	<1 μM	71	<10 μM
50	<1 μM	6	<0.5 μM
51	<5 μM	73	<5 μM
52	<1 μM	74	<5 μM
53	<1 μM	75	<5 μM
54	<5 μM		

Mouse #456 is a *Ptc*-knockout heterozygote that received UV irradiation for 6 months. The mouse developed many small BCC lesions, which were blue after X-gal staining. The mouse was sacrificed and the skin was excised with a 2 mm skin punch. Those skin punches were then cultured for 6 days. Comparing to vehicle (DMSO), compound A can decrease the number and size of BCC lesions (blue spots in the picture). This experiment suggests that compound A is able to inhibit murine BCC lesions in mouse #456.

In yet another experiment, E12.5 old *ptc-1 (d11) lacZ* lungs were harvested and transgenic embryos identified by lacZ detection using tails. Lung explants were grown submerged in mouse explant medium (DMEM based, additives optimized for the culture of mouse lungs) for 48 hrs, fixed in lacZ fixative, rinsed and stained for lacZ O/N at 37 °C. Control tissue was untreated, while test tissue was treated with compound A. Strong *lacZ* expression can be observed in distal and proximal mesenchyme. Treatment with compound A leads to significantly decreased reporter gene expression, as evidenced especially by the weak signal surrounding the distal branching tips of the growing lung epithelium.

Example 5: Bladder cancer

Cytogenetic and mutational data suggest *hedgehog* activation plays a causative role in bladder cancer

The cytogenetic and molecular alterations found in bladder cancer are heterogeneous. In establishing the primary, specific mutations in cancers, it is often useful to examine near-diploid

cancers, which do not yet have complex, multiple chromosome changes accompanied by hyperdiploidy. Gibas et al., found monosomy of chromosome 9 in 4 out of 9 cases of transitional cell carcinoma of the bladder (Gibas et al. (1984) Cancer Research 44:1257-1264). In three of these, the karyotype was near diploid, and in one, monosomy 9 was the only abnormality observed. Therefore, monosomy of chromosome 9 may initiate malignant transformation in a subgroup of such cancers.

More evidence that this change appears as an early event was presented by two other group who reported that deletions of chromosome 9 are the only genetic changes present frequently in superficial papillary tumors (Dalbagni et al. (1993) Lancet 342: 469-471). In fact, 9q deletions are estimated to occur in approximately 60-70 percent of bladder tumors (Cairns et al. (1992) Oncogene 8: 1083-1085; Dalbagni et al., *supra*). One study reported that deletion of 9q22 occurs in 35% of informative cases (Simoneau et al. 1999). The *hedgehog* signaling pathway component *patched-1* is located on 9q22.

LOH of all other chromosomes is infrequent (less than 10%) in low-grade, non-invasive cancers. Likewise, alteration in bladder-cancer associated oncogenes (ERBB2, EGFR) are also rare in superficial, low-grade tumors (Cairns et al., *supra*).

On the basis of these cytogenetic findings, the following model for bladder carcinogenesis has been proposed: Initiation occurs by deletion of tumor-suppressor genes on chromosome 9, leading to superficial papillary or occasionally flat tumors, a few of which may then acquire further mutations (e.g., p53) and progress to invasion.

Three groups observed trisomy 7 in a low percentage of bladder cancers (Sandberg, *supra*; Berger et al. *supra*; Smeets et al., *supra*). Shh, which according to our own experiments continues to be expressed in bladder epithelium throughout adult life, localizes to chromosome 7. Berger et al. also observed deletions of 10q24, the locus of su(fu) (Berger et al (1986) Cancer Genetics and Cytogenetics 23: 1-24). Likewise, Smeets et al. suggested that 10q loss may be a primary event in the development of bladder cancer (Smeets et al. (1987) Cancer Genetics and Cytogenetics 29: 29-41).

This data suggests mechanisms by which the baseline expression of *hedgehog* signaling present in the adult bladder epithelium may be increased, thus leading to increased proliferation of urothelial cells. This hypothesis is supported by the cytological data, as well as by the finding

of McGarvey et al. that described *ptc-1, smo* and *gli-3* expression in normal human urothelium and two transitional cell carcinoma lines (McGarvey et al. (1998) Oncogene 17: 1167-1172).

Hedgehog signaling was examined in the mouse bladder, and found to be present in normal bladder. In *Ptc-lacZ* transgenic newborn mice (*ptc-1 (d11) lacZ*), *LacZ* expression can be detected in the proliferating urothelial cells of the bladder epithelium, and more weakly, in adjacent mesenchymal cells (Figure 16A). Additional in situ hybridization analysis of adult mouse bladder indicates expression of *gli-1* in the bladder epithelium, and specifically in the proliferating urothelial cells (Figure 16B).

METHODS: For lacZ staining, *ptc-1 (d11) lacZ* bladder was harvested from the transgenic newborn mouse pups identified by lacZ detection using tails. Bladders were fixed in lacZ fixative, rinsed and stained for lacZ O/N at 37 °C, then processed for standard histology. Sections were counter-stained with eosin. For *in situ* hybridization, sections of paraformaldehyde-fixed, paraffin-embedded tissue were cleared, re-hydrated, digested with proteinase K, acetylated and hybridized with [33P]-labeled *gli*-1 RNA probe over night. After high stringency post-hybridization washes, slides were dipped in photo-emulsion, incubated for up to three weeks, developed, and imaged using dark field illumination. Dark-field signals were filled in with artificial color (red) and superimposed with bright-field images.

***Hedgehog* signaling in bladder cancer**

Hedgehog signaling and *hedgehog* pathway gene expression was analyzed in a human bladder cancer, and in several bladder cancer cell lines. Gene expression in these tissues was measured using Quantitative Real-Time PCR (Q-RT-PCR). These results are summarized in Figures 17-19, and demonstrate that *hedgehog* pathway genes are expressed in bladder cancer cell lines.

Figure 17 demonstrates that *shh* expression is increased 12-fold and *gli-1* expression is increased 2.5 fold in a bladder tumor sample when compared to normal adult bladder. Figure 18 examines *shh* and *gli-1* expression in eight human bladder cancer cell lines, and Figure 19 examines expression of *shh, ptc-1, smo, gli-1, gli-2, and gli-3* in the same eight human bladder cancer cell lines. These results indicate that components of the *hedgehog* pathway are expressed in eight out of eight cell lines examined.

METHODS: Experiment 1 (Figure 17) – evaluation of *hedgehog* signaling in a bladder tumor.

For Quantitative Real-Time Polymerase Chain Reaction (Q-RT-PCR) experiments, commercially available cDNA (Clontech) was amplified using an ABI Prism 7700 Sequence Detection System (TaqMan) from Perkin Elmer and gene-specific primers. The housekeeping gene GAPDH was used to normalize RNA concentration and PCR efficiency, and GAPDH primers were added to the same reactions. Since probes for both genes are labeled with different fluorophores, the specific signal and that of GAPDH can be detected in the same tube. Signal intensities were calculated using the algorithms provided in Sequence Detector v1.7, the software provided by the manufacturer.

Experiment 2 (Figures 18-19) –*hedgehog* signaling in eight bladder cancer cell lines.

Bladder cancer cell lines were purchased from ATCC (American Type Culture Collection) and maintained as recommended in the product description. At confluency, cells were rinsed and switched to medium containing 1% serum, a treatment that increases *hedgehog* signaling. Cells were then grown 2 more days, collected in Trizol (GIBCO-BRL) and RNA isolated according to the manufacturer's protocol. The RNA was then transcribed into first strand cDNA according to standard protocols, and amplified using an ABI Prism 7700 Sequence Detection System (TaqMan) from Perkin Elmer and gene-specific primers. The housekeeping gene GAPDH was used to normalize RNA concentration and PCR efficiency, and GAPDH primers were added to the same reactions. Since probes for both genes are labeled with different fluorophores, the specific signal and that of GAPDH can be detected in the same tube. Signal intensities were calculated using the algorithms provided in Sequence Detector v1.7 , the software provided by the manufacturer.

In vitro assay to examine *hedgehog* signaling in bladder cancer cell lines

The expression of components of the *hedgehog* signaling pathway in the eight bladder cancer cell lines examined suggested that *hedgehog* signaling is active in bladder cancer cells. However the gene expression observed may not be indicative of functional signaling. To assess whether functional *hedgehog* signaling occurs in bladder cancer cell lines, a gli-Luc in vitro assay was used. This assay is summarized schematically in Figure 20. Briefly, 10T ½ (S12) fibroblasts expressing a luciferase reporter gene responsive to *hedgehog* serve as an indicator of *hedgehog* signaling. When these cells are contacted with functional *hedgehog* protein, the *hedgehog* signaling pathway is activated in the S12 cells, and luciferase is expressed. In the experiments presented here, S12 cells are co-cultured with bladder cancer cells. If the bladder

cancer cell line secretes functional *hedgehog* protein, luciferase expression will be activated in the adjacent S12 cells.

Figure 21 shows luciferase induction in S12 cells alone, and in S12 cells co-cultured with three bladder cancer cell lines. Two of the three cell lines examined induced expression of luciferase in S12 cells indicating that these bladder cancer cell lines secrete functional *hedgehog* protein.

To confirm the specificity of this activation of *hedgehog* signaling by bladder cancer cell lines, S12/RT-4 co-cultures were treated with the Shh blocking antibody (5E1). Figure 22 demonstrates that 5E1 treatment of co-cultures inhibits expression of luciferase in S12 cells with an IC_{50} of 85ng/ml and an IC_{90} of 500ng/ml. It should be noted that this model also provides a means for evaluating the in vitro efficacy of other *hedgehog* antagonists including small molecule and polypeptide antagonists.

Hedgehog signaling in an in vivo mouse bladder tumor model

Injection of bladder tumor cells into nude mice induces tumor formation. Based on the ability of the Shh antibody 5E1 to inhibit *hedgehog* signaling in the in vitro gli-Luc assay described in detail above, the ability of 5E1 to inhibit bladder cell tumor growth in vivo was examined. Briefly, nude mice were injected subcutaneously with 10^7 RT-4 cells. The mice were divided into two groups and treated with either 5E1 or with a control IgG antibody. Figures 23 and 24 show that treatment with 5E1 significantly decreased the size of the tumor in comparison to treatment with the IgG control. It is important to note that due to the procedure used in this particular experiment (injection of tumor cells with Matrigel) the tumors start out with an average size of 100mm3 due to the Matrigel matrix (=100µl injection volume). Matrigel is a liquid when kept on wet ice, but solidifies upon injection. Thus, the average tumor size in the 5E1 group at the end of the experiment is roughly equal to that at the beginning of treatment. Results are highly statistically significant (Student's t-test: $p=0.017$). It should be noted that this model also provides a means for evaluating the in vivo efficacy of other *hedgehog* antagonists including small molecule and polypeptide antagonists.

In addition to evaluating the effect of 5E1 treatment on tumor size, expression of *gli-1* in both the RT-4 tumors and in the surrounding tissue was also evaluated. 5E1 treatment decreased expression of *gli-1* in both the RT-4 tumors and in adjacent tissue (Figure 25). This finding is

significant because the in vitro experiments outlined above indicate that these *hedgehog*-expressing cells can activate *hedgehog* signaling in adjacent cell. Given the complex nature of cancer progression, it is possible that *hedgehog* signaling influences cancer both directly and indirectly. The indirect effects may include the induction of proliferative factors, angiogenic factors, or anti-apoptotic factors, to name a few. The induction of such factors may occur within the cancer cells themselves or in adjacent cells. Thus, the demonstration that a *hedgehog* antagonist 5E1 can inhibit *hedgehog* signaling in both cancer cells and in surrounding cells has significant implications.

METHODS: Exponentially growing RT-4 cultures were trypsinized, spun down, and resuspended in a small volume of culture medium. The proportion of viable tumor cells was determined by trypan blue exclusion. 107 cells/animal were resuspended in 100μl Matrigel (a commercially available preparation of basement membrane components) and injected subcutaneously in the right side of the flank of 6-8 week-old athymic male BALB/c nu/nu nude mice. Treatment was begun the day after injection of the cells. Mice were divided into two groups containing 16 animals/group. The control group (IgG control antibody) and the 5E1-treated group were injected 3x/week intraperitoneally with 10mg/kg antibody. Tumors were measured 2X/week by caliper in 2 dimensions and measurements converted to tumor mass using the formula for a prolate ellipsoid (axb2x/2). As noted above, in this particular example the tumors were injected in combination with Matrigel. Therefore, the tumors have an initial size of 100 mm^3 and the inhibition of tumor size observed following 5E1 treatment is nearly a complete inhibition of tumor growth.

Expression of *gli-1* was measured using Q-RT-PCR as described throughout the application.

The inhibition of tumor growth by the *hedgehog* antagonist 5E1 supports the utility of the claimed invention. It is expected that antagonism of *hedgehog* signaling using a range of agents would have similar effects in decreasing tumor growth, and the efficacy of any candidate compound could be easily assessed using the in vitro and in vivo methods described above.

<u>*Example 6: Prostate Cancer*</u>

Hedgehog signaling plays an important role in normal prostate development. *Sonic hedgehog* is required for prostate growth, and expression of Shh is strongly correlated with prostate ductal branching (Podlasek et al. (1999) <u>Developmental Biology</u> 209: 28-39). Recent

evidence supporting the essential role of shh in proper prostate branching demonstrates that treatment of embryonic prostate with the *hedgehog* antagonist cyclopamine inhibits growth and branching (W. Bushman, unpublished result). Additionally, the maintenance of low levels of *hedgehog* signaling in the adult mouse prostate suggests additional roles for *hedgehog* signaling beyond this early role in the initial growth and branching of the embryonic prostate.

Recent studies have examined the correlation between the expression of components of the *hedgehog* pathway and prostate cancer. These results show a correlation between increased expression of *shh* and/or *gli-1* and prostate cancer. Additional cytological data supports the idea that mis-regulation of the *hedgehog* pathway plays a role in prostate cancer. Two studies have described deletions of a fragment of chromosome 10 containing the Su(fu) locus in prostate cancers (Carter et al. (1990) PNAS 87: 8751-8755; Li et al. (1997) Science 275: 1943-1947). Given the evidence in the literature suggestive of a role for *hedgehog* signaling in prostate cancer, *hedgehog* signaling in several prostate cancer cell lines was examined. Additionally, the ability of *hedgehog* antagonists to decrease activation of *hedgehog* signaling in prostate tumor cell lines was demonstrated. These results suggest that, like in bladder cancer cells, antagonism of *hedgehog* signaling has utility in decreasing growth and proliferation of prostate cancer cells.

***Hedgehog* signaling in prostate cancer**

Expression of *shh* and *gli-1* in both human prostate cancer samples and in commercially available prostate cancer cell lines was examined. Figure 26 shows in situ hybridization analysis of human prostate cancer samples, and demonstrates the abundant expression of *shh*. Similarly, Figure 27 demonstrates high levels of *gli-1* expression in prostate cancer cells as measured by Q-RT-PCR. Finally, Figure 28 examined expression of both *shh* and *gli-1* by Q-RT-PCR in three commercially available prostate cancer cell lines. These results indicate *hedgehog* signaling occurs in all three commercially available cell lines.

METHODS: In situ hybridization: Paraformaldehyde-fixed tissue is cryo-sectioned into 30μm sections, digested with proteinase K, hybridized overnight with digoxigenin-labeled RNA probe. After high stringency post-hybridization washes, sections are incubated with an anti-digoxigenin antibody which is labeled with alkaline phosphatase. The signal is visualized by addition of BM purple, a commercially available chromagen solution that reacts with the alkaline phosphatase to form a purple precipitate.

Prostate cancer cell lines were purchased from ATCC (American Type Culture Collection) and maintained as recommended in the product description. At confluency, cells were rinsed and switched to medium containing 1% serum, a treatment that increases *hedgehog* signaling. Cells were then grown 2 more days, collected in Trizol (GIBCO-BRL) and RNA isolated according to the manufacturer's protocol. The RNA was then transcribed into first strand cDNA according to standard protocols, and amplified using an ABI Prism 7700 Sequence Detection System (TaqMan) from Perkin Elmer and gene-specific primers. The housekeeping gene GAPDH was used to normalize RNA concentration and PCR efficiency, and GAPDH primers were added to the same reactions. Since probes for both genes are labeled with different fluorophores, the specific signal and that of GAPDH can be detected in the same tube. Signal intensities were calculated using the algorithms provided in Sequence Detector v1.7 , the software provided by the manufacturer.

In vitro assay to examine *hedgehog* signaling in prostate cancer cell lines

The expression of components of the *hedgehog* signaling pathway in prostate cancer samples and cell lines suggests that *hedgehog* signaling is active in prostate cancer. However the gene expression observed may not be indicative of functional signaling. To assess whether functional *hedgehog* signaling occurs in prostate cancer cell lines, the gli-Luc in vitro assay was employed. This assay was summarized above, and is represented schematically in Figure 20. Briefly, 10T ½ (S12) fibroblasts expressing a luciferase reporter gene responsive to *hedgehog* serves as an indicator of *hedgehog* signaling. When these cells are contacted with functional *hedgehog* protein, the *hedgehog* signaling pathway is activated in the S12 cells, and luciferase is expressed. In the experiments presented here, S12 cells are co-cultured with prostate cancer cells. If the prostate cancer cell line secretes functional *hedgehog* protein, luciferase expression will be activated in the adjacent S12 cells.

Figure 29 shows no induction of luciferase in S12 cells cultured alone, or in S12 cells cultured with PZ-HPV-7 (normal) cells. However, luciferase induction is observed when S12 cells are cultured with any of three prostate cancer cell lines: 22Rv1, PC-3, or LNCaP. This result indicates that these prostate cancer cell lines secrete functional *hedgehog* protein.

To confirm the specificity of this activation of *hedgehog* signaling by prostate cancer cell lines, S12/prostate cancer co-cultures were treated with the Shh blocking antibody (5E1). Figure 30 demonstrates that 5E1 treatment of co-cultures inhibits expression of luciferase in S12 cells. METHODS: S12 cultures and co-cultures, and luciferase assays were performed as detailed above.

<u>*Example 7: Benign Prostatic Hyperplasia (BPH)*</u>

As detailed above, *hedgehog* signaling appears to have both an important role in early prostate patterning, and a role in maintenance of the adult prostate. Although prostate cancer is one potential affect of misregulation of *hedgehog* signaling in the adult prostate, another common condition of the prostate that seems to correlate with *hedgehog* expression is benign prostatic hyperplasia (BPH).

BPH is a disease of the central prostate, and is characterized by increased smooth muscle around the prostatic urethra. Interestingly, *shh* is expressed in a gradient in the adult prostate with highest expression in the central zone of the prostate. Additionally, *shh* is involved in smooth muscle differentiation in other tissues including the gut and lung (Apelqvist et al. (1997) Current Biology 7: 801-804; Pepicelli et al. (1998) Current Biology 8: 1083-1086). This evidence identified *hedgehog* signaling as a good candidate for involvement in the etiology of BPH. Finally, transcription of *shh* is increased by exposure to dihydro-testosterone (DHT) (Podlasek et al., *supra*). This is significant because the concentration of 5-alpha-reductase, an enzyme which converts testosterone to DHT, is elevated in BPH stroma (Wilkin et al. (1980) <u>Acta Endocrinology</u> 94: 284-288). This data suggests that mis-regulation of *hedgehog* signaling may be involved in BPH, and thus that the present invention provides utility for the treatment of BPH.

Hedgehog signaling in BPH

Expression of *sonic hedgehog* and *gli-1* expression in human BPH samples was examined. Figures 31 and 32 show in situ hybridization analysis of human BPH samples, and demonstrate that both *shh* and *gli-1* are abundantly expressed in BPH. Furthermore, Figure 33 demonstrates that *shh* is not ubiquitously expressed throughout the prostate, but is instead present in a gradient with the highest level of both *hedgehog* and *ptc-1* transcripts present in the proximal central zone of the prostate.

Additionally, the expression of *shh* and *gli-1* by Q-RT-PCR was analyzed. Figure 34 shows that both *shh* and *gli-1* are expressed in BPH samples. Expression of *shh* and *gli-1* in basal cell carcinoma (BCC) samples is provided for comparison. These results demonstrate that *gli-1* is expressed in BPH samples at a level similar to that found in a cancer type known to be caused by a *hedgehog* pathway mutation. Finally, Figure 35 shows the expression of *shh* and *gli-1* in BPH cell lines, and compares expression to that observed in BCC, prostate cancer cell lines, and normal prostate fibroblasts. Note that *gli-1* is expressed at similar levels in both BPH cell lines and in BCC samples. These results are suggestive of a role for *hedgehog* signaling in BPH and further suggests that antagonism of *hedgehog* signaling has significant utility in the treatment of BPH.

METHODS: In situ hybridization (Figures 31 and 33): Paraformaldehyde-fixed tissue is cryo-sectioned into 30μm sections, digested with proteinase K, hybridized overnight with digoxigenin-labeled RNA probe. After high stringency post-hybridization washes, sections are incubated with an anti-digoxigenin antibody which is labeled with alkaline phosphatase. The signal is visualized by addition of BM purple, a commercially available chromagen solution that reacts with the alkaline phosphatase to form a purple precipitate.

Radioactive In situ hybridization (Figure 32): Briefly, 7mm sections of paraformaldehyde-fixed, paraffin-embedded tissue containing large basal cell islands are cleared, re-hydrated, digested with proteinase K, acetylated and hybridized overnight with ^{33}P- labeled RNA probes. After high stringency post-hybridization washes, slides were dipped in photo emulsion and incubated in the dark for 14 days at 4°C. After developing, slides were counter-stained with hematoxylin and eosin and imaged using dark-field illumination. Dark-field images were converted to red artificial color and superimposed with bright-field images.Q-RT-PCR: Samples were collected in Trizol (GIBCO-BRL) and RNA isolated according to the manufacturer's protocol. The RNA was then transcribed into first strand cDNA according to standard protocols, and amplified using an ABI Prism 7700 Sequence Detection System (TaqMan) from Perkin Elmer and gene-specific primers. The housekeeping gene GAPDH was used to normalize RNA concentration and PCR efficiency, and GAPDH primers were added to the same reactions. Since probes for both genes are labeled with different fluorophores, the specific signal and that of GAPDH can be detected in the same tube. Signal intensities were calculated using the algorithms provided in Sequence Detector v1.7 , the software provided by the manufacturer.

<u>Example 8: Antagonism of hedgehog Signaling in Colon Cancer</u>

The growth of tumors is a complex process that requires proliferation, angiogenesis, the inhibition of cell death, and many other complex interactions between the cancer cells and the surrounding tissue. An additional mechanism by which *hedgehog* signaling may influence tumor growth and progression is through the induction of factors that enhance proliferation, angiogenesis, and the inhibition of cell death. For example, *sonic hedgehog* has been shown to induce VEGF in fibroblasts. Thus, the use of *hedgehog* antagonists may prevent *hedgehog* signaling from inducing factors that promote tumor formation, and therefore inhibit tumor formation or progression.

To help address this model, the ability of the antagonistic *hedgehog* antibody 5E1 to inhibit tumor growth in mice injected with a combination of *hedgehog* expressing colon cancer cells and fibroblasts was investigated. Two experiments were performed to assess the effects of 5E1 treatment on tumor size in mice injected with *hedgehog* expressing colon cancer cells. In the first experiment, treatment with 5E1, or PBS control, was initiated on the same day as injection with the tumor cells. The results are summarized in Figure 36, and demonstrate that treatment with 5E1 significantly decreases tumor size, weight, and rate of growth in comparison to that of mice treated with PBS (Figure 36). The experiment was performed using two separate colon cancer cell lines with similar affects.

In the second experiment, treatment with 5E1 was delayed until the eleventh day of tumor growth. The results are summarized in Figure 37, and demonstrate that treatment with 5E1 significantly decreases the size and rate of growth of the tumor when compared to control mice (Figure 37). The experiment was performed using two separate colon cancer cell lines with similar affects.

These results demonstrate the utility of *hedgehog* antagonists in the inhibition of proliferation and growth of cancer cells. Additionally, this model provides an in vivo method for easily evaluating the efficacy of candidate *hedgehog* antagonists.

METHODS: Experiment 1. Twenty nude mice were injected subcutaneously with a combination of 10^6 HT-29 cells (a Shh expressing colon cancer cell line) and 10^6 10T ½ cells (a fibroblast cell line) in a volume of 100μl. The mice were randomized into two groups. Group A was treated with PBS, and group B was treated with 5E1. The treatments were initiated on the same day as

injection of the tumor cells. Treatment was administered IP, 3 times/week over a period of thirty days, and at a dose of 6mg/kg. Additionally, this experiment was carried out under an identical protocol using another Shh expressing colon cancer cell line (Colo205) with similar results. Experiment 2 – delayed administration. Twenty nude mice were injected subcutaneously with a combination of 10^6 HT-29 cells (a Shh expressing colon cancer cell line) and 10^6 10T ½ cells (a fibroblast cell line) in a volume of 100μl. The mice were randomized into two groups. Group A was treated with PBS, and group B was treated with 5E1. Treatment was initiated after the tumor had grown to day 11. Such tumors had a volume of approximately 90-210 mm^3. Treatment was administered IP, 3 times/week over a period of twenty-nine days (until day 40 of total tumor growth), and at a dose of 6mg/kg. Additionally, this experiment was carried out under an identical protocol using another Shh expressing colon cancer cell line (Colo205) with similar results.

Example 9: Drug Screens

The foregoing examples present both in vitro and in vivo models for examining the effects of hedgehog antagonist on cell proliferation. The models provide assays for testing a range of antagonistic agents for the ability to inhibit cell growth and proliferation. Such screens can be used in initial assays to identify lead compounds, and can also be used to evaluate the relative efficacies of candidate compounds.

Antagonistic agents that can be analyzed in this way include small molecules, blocking antibodies, antisense oligonucleotides, and polypeptides. These agents may interfere with hedgehog signaling at any point along the signal transduction pathway. For example, preferred agents may interact with *hedgehog, patched-1, or smoothened.* Additional preferred agents may interact with an intracellular component of the hedgehog pathway including *gli-1, gli-2, or gli-3.*

The in vitro and in vivo methods described above are not specific for the cancer cell lines explicitly described herein. Any cell type or cell line could be similarly tested, and these methods could be easily used to assess the ability of hedgehog antagonists to inhibit tumor growth and proliferation in other types of cancer cells. Additionally, the in vitro assay could be employed to analyze hedgehog signaling and the ability of hedgehog antagonists to block hedgehog signaling in other non-cancerous hyperproliferative cell types. For example, hyperproliferative conditions include many other classes of disorders including skin maladies such as psoriasis. The effects of

candidate hedgehog antagonists on these cell types can be easily assessed using the methods described here.

All of the references cited above are hereby incorporated by reference herein.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.